Spring 1-1-2011

Production, Optimization and Characterization of Pharmaceutical and Excipient Powders Produced by Carbon Dioxide-Assisted Nebulization with a Bubble Dryer (CAN-BD)

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by

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B.S., Linfield College, McMinnville OR, 2004

A thesis submitted to the

Faculty of the Graduate School of the

University of Colorado in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

Department of Chemistry and Biochemistry

2011

This thesis entitled:

Production, Optimization and
Characterization of Pharmaceutical and Excipient Powders Produced by Carbon Dioxide-Assisted Nebulization with a Bubble Dryer (CAN-BD)

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Date: _______________

The final copy of this thesis has been examined by both the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.

David H. McAdams (Ph.D. Chemistry and Biochemistry)

Production, Optimization and Characterization of Pharmaceutical and Excipient Powders Produced by Carbon Dioxide-Assisted Nebulization with a Bubble Dryer (CAN-BD)

Thesis directed by Professor R.E. Sievers

Vaccination is frequently acknowledged as the invention most beneficial to human development. In the past 50 years vaccination has advanced greatly in terms of consistency and efficacy, but, in order to improve accessibility of vaccines to underserved populations in the developing world, more research must be conducted to improve the safety of delivery, affordability and stability of vaccines. This doctoral dissertation work has centered on the production of stable, dry powder vaccines and pharmaceuticals for inhalation. A principal goal has been to prepare consistent dry powders of measles vaccine for delivery by inhalation with activity comparable to commercially available products. Samples of vaccines received from collaborators at the Serum Institute of India have been analyzed and characterized to ensure consistency. This research has also uncovered a metastable crystal polymorph of *myo*-inositol (the primary excipient) as verified by powder X–ray diffraction and calorimetric studies. Research has also been conducted on the development and optimization of a dry powder, protein subunit Human Papillomavirus vaccine for delivery by inhalation, sublingual pellet or reconstitution for injection. One can hope that this research will lead to the advancement of vaccination and pulmonary delivered pharmaceutical powders, aiding in the reduction of global mortality and morbidity due to vaccine preventable disease.

This Dissertation is dedicated to my Father, Mother and Sister (David, Nita and Nellie McAdams). Thank you so much for being there for me for the past 7 years of grad school. You provided the support I needed from the outside world and I never would have made it here without the support and love you gave me. This is for you.

Acknowledgements

This Dissertation has been a long and hard-earned achievement and I never would have made it to this point alone. The following is a list of just some of the acknowledgements I owe to all those who have helped me throughout my graduate career.

I would like to thank my teachers. I never would have been able to advance this far in science without a terrific start due to my high school teachers George Penk and Walt Looney. My knowledge was formed and applied by my Linfield College Profs: Liz Atkinson, Robert Wolcott and Brian Gilbert. The advanced skills and theory I learned in grad school guided me through my research and I owe these to the CU Chem Department Professors.

I would like to thank my research advisor, Dr. Bob Sievers for his guidance and support throughout my studies. Dr. Steve Cape has always been there to guide my labwork and provide excellent direction in my research. I would like to thank all the former labmates and CU researchers I have worked with: Jaime Manion, Jessica Best, Dr. Liz Gersh, Dr. Robert Garcea, Molly Larson and Dr. Mark Hernandez. The staff and researchers at Aktiv-Dry have provided endless support and I would like to thank them all especially Brian Quinn, Pankaj Pathak and Scott Winston for their feedback and support.

Graduate school would never work without a life outside of lab and I would like to single out my "Man Brothers": Anthony Morfa, Jesse Marcum, Patrick Veres, Will Rance and Stephen Ponicsan (with "man sister" Elisa) for fishing tips, quality stories and excessive amounts of bacon. I would like to acknowledge my friends, roommates, CU staff and coffee buddies from Boulder, Denver and Sweden. I would never be able to fit all of your names in here, but you know who you are, and I want you know I am eternally grateful.

Contents

Chapter I Introduction

1.1 Vaccination

The earliest recorded variant of vaccination was practiced in China in the 17th century. The practice known as variolation was performed by placing a piece of cotton containing pus from a smallpox pustule into the nostril of a healthy child (Leug 1996). Presentation of the smallpox virus, compromised by the source patient's immune response, resulted in an immune response in the recipient with reduced severity resulting in immune protection from smallpox infection in the future. Another method of delivery was to blow powdered smallpox squama into the nostril of a healthy child using a small silver tube. Interestingly, this method "was said to be convenient for bringing the technique to remote areas" (Leung 1996), much like the motivation behind our development of an inhaled measles vaccine.

Fortunately vaccination has been significantly developed since the inhalation of scabs and pus. Vaccination is consistently cited as one of the inventions most beneficial to human development. By providing preemptive protection, vaccines have greatly improved our quality of life, reducing the morbidity and mortality historically related to "unavoidable" diseases. Vaccination results in the development of a natural immune response in the recipient, specific to the disease of interest. While there are many different types of vaccines based on the identity of the antigen presented (DNA, Killed, Toxoid conjugated, etc.), the vaccine types that will be concentrated on in this dissertation will be: subunit and live-attenuated.

The active ingredients in subunit vaccines are made up of deactivated or artificially produced immunogenic material related to the disease of interest. In order to elicit an immunogenic response to the disease, these materials usually mimic the antigens targeted by the immune system in an infection by the wild type disease agent, such as viral surface proteins. Successful subunit vaccines generally require the addition of an adjuvant, or material intended to induce an increased immune response by, among other pathways, recruiting immune cells to the site of injection and promoting presentation of the antigen to the immune system. Subunit vaccines can have a greater range of stability, due to the simplicity of the immunogenic components, but the adjuvant material included in the formulation may limit the stability of the entire formulation. For instance, it is commonly observed that when a liquid formulation containing an alum-adjuvated antigen is frozen, the alum adjuvant frequently dissociates from the material, reducing the activity of the vaccine (Maa, Zhao et al. 2003). It is for this reason that Hepatitis B and Gardasil, among many other liquid vaccines, cannot be lyophilized and are required to be stored between 2 and 8 °C. These liquid vaccines cannot be frozen, as they would loss activity, and require advanced temperature regulation during storage to remain viable.

Live-attenuated vaccines use similar or altered versions of the disease agent that are still able to infect living cells and replicate, but have a diminished ability when compared to the wild type disease agent. The living-attenuated agent is delivered and allowed to replicate, increasing the amount of immunogenic material in the recipient. The immune system is able to neutralize the agent without the development of a full infection, in the process developing the ability to immunogenically respond to subsequent infections with the attenuated and wild type disease agent. The first successful vaccine was a live virus vaccine for smallpox developed by Edward Jenner in 1798 (Artenstein 2010). Before Jenner, the practice of variolation, or inoculation with pus from another patient suffering from smallpox, had been widely, but not commonly, used in England and abroad to provide protection from smallpox. Based on observations that milkmaids were less susceptible to smallpox, Jenner hypothesized that exposure to cowpox resulted in protection from the smallpox virus. By placing bovine tissue infected with cowpox over an open cut on a human patient, the patient was essentially infected with cowpox virus. This virus was specifically adapted to infect bovine cells, and defend itself from the bovine immune system, so the human immune system was able to quickly neutralize this antigen and in doing so add a pathway of response to the “memory” of the immune system specific to this antigen. The similarities between the cowpox and smallpox viruses allowed the human immune system to respond with immunogenic neutralization and protection from the extensive viral replication of wild type smallpox (Alzhanova and Fruh 2010). The invention of the smallpox vaccine by Jenner and the vaccine campaigns that followed greatly increased the quality of life and reduced the mortality due to endemic smallpox. With further development and advancement of the smallpox vaccine and well organized vaccination campaigns, natural smallpox infection was declared to be eradicated in 1980. Even with the reported eradication of smallpox , there is still active research on smallpox vaccine development to respond to potential biological weaponization or some other pathway of smallpox reemergence (Henderson 2011) . Vaccines have been shown to greatly improve the quality of life of recipients but in practice, vaccination also has the potential to cause some health problems and death, in addition to controversy over vaccine delivery.

As with any medical procedure involving the transfer of infected material to the bloodstream, vaccination always carries some inherent dangers. In performing the Jenner vaccination procedure, particularly with the medical and sanitation practices of the 18th and 19th century, occasional infection of the inoculating material with smallpox and other infective agents was observed (Hopkins 2002). Vaccinations with live-attenuated agents are infections and are associated with symptoms, such as rash, nausea and other negative health conditions that are similar to, but of less severity than, infection with the wild type disease (Siegrist 2007; Artenstein 2010). In recipients with compromised immune systems, vaccination may not be as effective and may even cause health risks (Isaguliants 2007).

Other difficulties arise in the stability and potential for contamination of the vaccine material (Sood, Kumar et al. 1995). In order to prevent cross contamination or the replication of other infective agents it is recommended that multidose vials of liquid vaccines be discarded after 6 hours and multidose vaccines are required to be formulated with preservatives (Clements, Larsen et al. 2004) .

In 1998, a British gastroenterologist, Andrew Wakefield, and 12 other authors published a paper in the Lancet suggesting a connection between the delivery of the Measles, Mumps and Rubella (MMR) vaccine in infants and the subsequent diagnosis of autistic symptoms. Most anti-vaccination advocates attribute the observed autistic symptoms to the presence of thimerosal, an organomercury containing preservative commonly added to multidose vials of vaccine, in the MMR vaccinations. This is an interesting assertion, as the MMR vaccine does not and has never contained thimerosal as a preservative (FDA 2010). When evidence of conflict of interest arose and the paper's findings were not supported by subsequent research, 10 of the 12 authors retracted the interpretation of the results, while Wakefield continued to maintain the purported connection between MMR vaccination and Autism. Despite the retraction of the paper by the Lancet and the denial of his right to practice medicine in the UK, Wakefield has continued to support his findings as verifiable and ethically obtained (Mnookin 2011).

In recent times, many vocal groups have arisen promoting the belief that childhood vaccination (particularly with thimerosal containing vaccines) is a cause of autism. Many prominent spokespeople in the anti-vaccination campaigns have arisen including celebrities Jim Carey and Jenny McCarthy, who, while not backed by extensive scientific research, assert that childhood and infant vaccination lead directly to autism. Average rates of immunization in Britain dropped from 92 % to 73 % in 2002 and remain below 85% in 2011 (Mnookin 2011)In the wake of anti-vaccination advocacy, intentionally unvaccinated individuals may undermine the "herd immunity" of a generally immune population.

The extensive vaccination of a population also introduces the added benefit of “herd immunity”. When a sufficient proportion of a population, thought to be 70% to 95%, shows immunity to a disease, individuals in that population susceptible to the disease are sheltered from infection by avoiding exposure to the infectious agent (Brown, Shanley et al. 2011). If a sufficient portion of the population rejects vaccination, reservoirs of the disease may appear in the general population which may lead to the infection of unimmunized infants and immunocompromised individuals. Full compliance with mass vaccination campaigns is required for the complete eradication of a disease.In a 2008 measles outbreak in San Diego, 75% of the cases were reported in intentionally unvaccinated individuals (Fine 1993).

The standard method of vaccine delivery is intramuscular (i.m.) injection with disposable syringes. This method consistently delivers the vaccine and does not require active participation on the part of the recipient, which makes it possible to deliver vaccines to infants. There are, however, inherent dangers associated with vaccination by injection. Contact with the blood of vaccine recipients introduces the possibility of transmission of blood-borne diseases to the patients, healthcare providers and anyone exposed to the needles after use. A 1999 study found that, in regions of the developing world, more than 50% of injections were unsafe and increase the risk of spreading HIV, hepatitis and other blood-borne pathogens, with an estimated 20% of the new cases of hepatitis B attributable to unsafe injections (Sugerman, Barskey et al. 2010). The use of needles also puts excessive risk on the healthcare providers, particularly in a mass vaccination campaign in areas with insufficient healthcare services. A 2008 study found that 12.5% of percutaneous injuries occur when recapping needles and 31.2% occur when healthcare workers do not have proper facilities for sharps disposal (Simonsen, Kane et al. 1999). This added danger can greatly discourage recruitment of skilled vaccine providers and result in the removal from service of healthcare workers infected with a blood borne disease. In order to avoid the inherent dangers of injection, and the risk and cost of safe disposal of sharps, our research concentrates on the development of dry powder vaccines for inhalation.

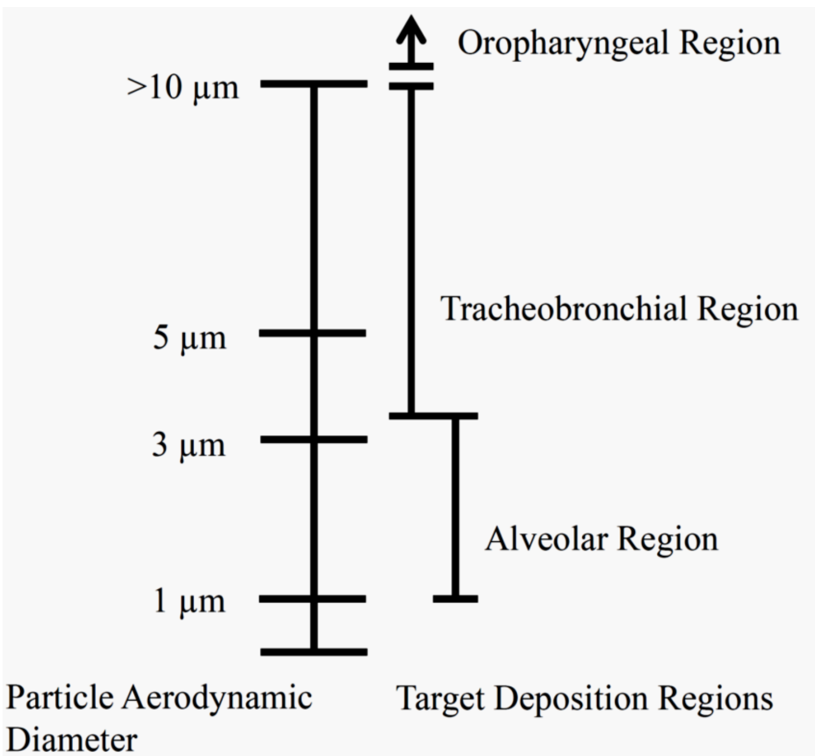
A recent review of possible innovations in measles vaccine delivery (Deuffic-Burban, Delarocque-Astagneau et al. 2011) has determined that delivery of measles vaccine as an inhalable dry powder may be the most promising new method of delivery, in terms of cost-savings. The study analyzed 4 different potential methods of vaccination: aerosolized liquid vaccine, jet injector, inhalable dry powder and DNA-based vaccine. These methods were analyzed based on the cost of dose per child and potential to reduce mortality and morbidity for the estimated >4.2 billion potential candidates for a new vaccine. The results of this study clearly distinguish inhaled dry powder measles vaccine as the method with the greatest potential for cost savings. Inhaled dry powder vaccine was estimated to result in a cost impact of -$689.4 million dollars over 40 years (compared to the -$154.1 million for aerosolized liquid, -$98.4 for jet injection and +$3,959 for DNA vaccine). This shows promise that an initial investment into research on a dry powder measles vaccine can result in a final product with a savings of $0.170 per dose, greatly improving the availability of this vaccine for mass vaccination campaigns.

1.2 Vaccination by Inhaled Particles

The lungs are well suited as a route of vaccination. The human lungs have a high surface area, the average human alveolar surface area is 143±12 m^2 (Garrison, Bauch et al. 2011), and extensive vasculature. The mucosal layer of the lung monitors inhaled antigens through the respiratory epithelium, alveolar macrophages and dendritic cells (Gehr, Bachofen et al. 1978). When an antigen is identified, a cascade of signals results in the release of proinflammatory cytokines which promote inflammation and recruitment of additional specific immune cells, such as neutrophils, Natural Killer (NK) cells, monocytes and memory T cells (Kohlmeier and Woodland 2009). After the initial defense against and clearance of the antigen or infecting virus by naïve immune cells, antigen-specific T and B immune cells are converted to long-lived memory immune cells, capable of responding quickly to a secondary infection. After the successful immune defense of the lungs a higher local concentration of memory T and B cells remain in the lung mucosal tissue (Piqueras, Connolly et al. 2006). While the number of antigen specific memory T cells in the lung tissue normalizes to the systemic concentration after several months, antigen-specific memory B cells remain at a higher concentration in lung tissue, or site of infection. These memory B cells localized in the lung tissue can respond quickly to a secondary infection and may secrete antigen -specific IgA, mucosa-specific immunoglobulin, to the mucous of the lung epithelia, leading to the immediate clearance or identification of infective agents (Kohlmeier and Woodland 2009). There is additional evidence that the route of infection and initial immune response may result in improved immune response on secondary infection or even subsequent infections by a different agent (Liang, Hyland et al. 2001). Initial infection of the lung may condition the local immune system, resulting in an improved heterologous immunity.

Due to the possibility of improved immune response and inherent safety of needle-free delivery, we have focused our research on developing vaccines delivered by inhaled dry particles. In order to effectively deliver our vaccines to the lungs we must carefully control the physical properties and size of the particles. The human respiratory system can be divided into the nasal-pharyngeal region, the upper airways, the lower airways and the alveoli. Inhaled particles will deposit differently in each of these separate regions based on particle size, shape, mass and momentum (Figure 1.2.1) (Chen, Fraire et al. 2003).

Inhaled particles larger than 10 µm in aerodynamic diameter (D_A), defined as the diameter of the particle if it were to be a perfectly spherical particle and made of a material with a density of 1 g/cm^3, generally deposit in the nasal-pharyngeal and upper airway regions due primarily to the impaction and interception methods of deposition. These particles are usually removed to the digestive tract by swallowing. At normal breathing rates (20 L/min) and higher, particles with D_A larger than 3 μm deposit by the primary mechanism of inertial impaction in the tracheobronchial region (upper and lower airways), with increased deposition of the larger particles (Hinds 1999). These particles deposit in the lung mucous and may be transferred by the mucosal elevator to the throat, where they will be transferred to the gastrointestinal system by swallowing. Particles with D_A values of 0.5 to 3 μm can deposit in the tracheobronchial region but generally penetrate the upper respiratory system to deposit in the alveoli by diffusion.



1.2.1 Figure deposition diagram

Ultimately, particles below 10 μm D_A reach the alveoli with varying amounts of attenuation due to deposition in the upper airways (Hinds 1999). The particle size range generally chosen for deposition in the alveolar region is between D_A = 1 and 5 μm, where deposition in the upper airways is minimized but particles are likely to deposit and not remain in suspension to be exhaled. Particles deposited in the alveolar region are not subject to the mucosillary elevator and have an increased residence time in the lungs. The primary methods of removal for these particles is by dissolution and transfer across the alveolar epithelia or by removal by macrophages or dendritic cells, which may result in an antigen-specific immune response. In order to produce powders within this particle size, particle engineering must be employed to use the correct formulation and process parameters for optimal vaccine particles.

Particle engineering is a relatively new area of research concentrating on the controlled formation of micro and nanoparticles for specific properties of size, shape, surface characteristics and internal composition (Hinds 1999). Particles can be described by their geometric diameter (D_g), as determined by microscopy techniques. A more valuable measure of particle size, for an inhaled pharmaceutical, is the aerodynamic diameter (D_A), which takes into account differences in the momentum of particles caused by density variability. The aerodynamic diameter of a spherical particle can be related to the geometric diameter by Equation 1.

$$D_A = \sqrt{\frac{\rho_P}{\rho*}} D_g \qquad (1)$$

Where ρ_P is the particle density and $\rho*$ is the unit density (1 g/mL) (Hinds 1999).

Aerodynamic diameter is determined by analysis of the momentum of the particles, such as time of flight measurements and selective impaction or impingement methods. These testing methods can be very dependent on the method of particle dispersion, as less powerful methods of dispersion may lead to the aerosolization of particle aggregates with larger observed D_A values.

In spray drying, the aerodynamic diameter can be related to the concentration of the initial stock solution (C_F) and the droplet diameter (D_D) by Equation 2 (Chow, Tong et al. 2007). This uses the assumption that the final particle is a solid, spherical particle.

$$D_A = \sqrt[6]{\frac{\rho_P}{\rho*}} \sqrt[3]{\frac{C_F}{\rho*}} D_D \qquad (2)$$

From Equation 2 it can be seen that initial droplet diameter is the primary deciding factor of the aerodynamic diameter of the dry particle with weaker relationships to stock concentration and particle density. In order to optimize the particle size, many methods decrease the stock concentration (as low as 0.01%). This increases the ratio of water/solvent to solid material which can result in high residual moisture in the dried particles and will require additional drying energy and temperatures, compared to higher stock concentrations, which may decrease the activity of thermally sensitive active ingredients. Higher concentrations (our standard concentrations are 10% mass/volume) produce more product for a given spray flow rate and have lower solvent removal energy requirements. Higher concentration feed stock can result in larger final particles, due to larger amounts of solute in the individual droplets, but particle size can be reduced by decreasing the size of the droplets with more energy added at atomization. For most methods of spray drying, scale-up can be more readily achieved with increasing the flow rate rather than greatly increasing the concentration. We have observed decreased particle sizes with lower concentration, but the relationship between aerodynamic diameter and concentration is complicated by the additional relationship between droplet size and solution viscosity/surface tension.

In a drying droplet, not all components will behave the same and component enrichment of the surface or core of the particle may occur (Vehring, Foss et al. 2007; Vehring 2008). In a drying spherical droplet, the surface enrichment (E_i) of component i, or the ratio of its concentration on the surface ($c_{s,i}$) to its concentration in the droplet bulk ($c_{m,i}$) can be estimated by Equation 3 (Vehring 2008).

$$E_i = \frac{C_{s,i}}{C_{m,i}} = \frac{e^{(0.5Pe_i)}}{3\beta_i} \qquad (3)$$

Where β_i is a function dependent on the Peclet number (Pe) and the Peclet number is a dimensionless value descriptive of the relative mobility of the component i in the drying solution. The Peclet number can be calculated based on the evaporation rate (κ) of the droplet and the diffusion rate for component i (D_i), Equation 4 (Vehring 2008).

$$Pe_i = \frac{\kappa}{8D_i} \qquad (4)$$

The diffusion rate for a given solute component in a drying droplet is heavily reliant on the size of that component. Large or insoluble molecules will have mobility in the feed solution compared to the evaporation rate for the droplet and thus will be "gathered" by, or concentrated on, the inward moving surface of a drying droplet. As the droplet shrinks and the surface area decreases, the material enriched on the surface may reach sufficient concentration to form a solid shell. The remaining solvent, with the remainder of the dissolved components, will continue to evaporate inside this outer shell. Depending on the diffussability of the vapor through this shell, the particle may burst to release the vapor, or allow the vapor to pass through the shell, creating a hollow or porous particle with a density lower than the bulk material. Some formulations intentionally include surface active (Vehring, Foss et al. 2007) or slowly diffusing nanoparticles (Vehring, Foss et al. 2007) to intentionally form large porous or hollow particles. Hollow particles can be beneficial to inhalable formulations as they may require less energy to disperse and may have the D_A sufficient to reach the deep lung but a D_g that is too large to allow phagocytosis by macrophages in alveoli, will result in an extended lifetime in the lungs (Vehring, Foss et al. 2007).

1.3 Spray Drying

Spray drying has been studied since the 1860s, but was extensively modified and improved during World War II with efforts to transport large amounts of foodstuffs over great distances (Lechuga-Ballesteros, Charan et al. 2008). In general use, such as the production of powdered milk and other food stuffs, spray drying removes most of the water from an aqueous solution or suspension of the material of interest. The dehydration of the material reduces the potential for microbial activity, increases the product stability and reduces the weight and volume of the final product (Tsapis, Bennett et al. 2002). The reduction of a material to small particles with large surface areas may also be desirable to increase the rate of dissolution of lower solubility materials (Tsapis, Bennett et al. 2002). Spray drying is the conversion of a fluid feed material to particulate form through the introduction of droplets of fluid feed into a gaseous medium. The spray drying process can be divided into three separate phases: atomization, drying in a gaseous medium and product collection.

1.3.1 Subsection 1.3.1 Atomization

Atomization is the process of forming a dispersion of the feed stock fluid in a gaseous medium. The fluid feed stock may consist of a solution or suspension of the material of interest in any fluid solvent or a fluidized or molten form of the material of interest. The most common earlier methods of atomization are: rotary, hydraulic nozzles, pneumatic nozzles and ultrasonic nozzles (Tsapis, Bennett et al. 2002).

Rotary nozzles use centripetal forces to produce an atomized spray of the feedstock. This is achieved by running the liquid feedstock over a spinning surface; spray is produced when the liquid is flung from the edge of the surface. This process is used for large scale spray drying and is easy to scale up, but produces large droplets, not conducive to obtaining final dried particles in the inhalable size range.

Hydraulic nozzles produce droplets by forcing a pure liquid feedstock through an orifice, as a jet, at high pressures/flow rates. Liquid break-up when exiting a jet can be described based on the dimensionless number Z' (The ratio of the Weber/Reynolds numbers).

$$Z' = \frac{\mu}{(\rho_1 \sigma d_n)^{1/2}} \qquad (5)$$

Where ρ_1= liquid density, d_n=jet diameter, σ=surface tension and μ=liquid viscosity. The ratio of the Z' number of a jet to the Reynolds number of the jet can describe the break-up pattern of the liquid exiting the jet with complete atomization occurring at high values of this ratio (Cal and Sollohub 2010). A low viscosity feedstock is required for this method and the final dried particles are usually in a broad size range, well above the desired size range for inhalation (Jangam 2011).

Pneumatic nozzles, or multi-fluid nozzles, operate by combining the liquid feedstock with a carrier gas. The friction between these two fluids causes the liquid disintegration of the feedstock. The mean droplet size (D_{DM}) produced by this method is very complex but can be approximated by Equation 6

$$D_{DM} = \frac{A}{(V_{rel} * \rho_a)^{\alpha}} + B\left(\frac{M_{air}}{M_{liq}}\right)^{\beta} \qquad (6)$$

Where A,B, α and β are variables dependent on nozzle design and liquid properties, V_{rel} is the relative velocity between air and liquid at the orifice, ρ_a is the density of the air and M_{air}/M_{liq} is the mass ratio of air to liquid (Srinarong, de Waard et al. 2011). From this it can be seen that for higher flow rates of carrier gas, smaller droplet size can be achieved. The air can be introduced to the liquid before or, more commonly, at the exit of the nozzle (Cal and Sollohub 2010). A wide variety of intricately engineered pneumatic nozzles have been produced to control the size range of droplets in the spray. Currently, pneumatic nozzles are the most common form of atomization used for the production of inhalable pharmaceutical particles.

Other variants of atomization exist, but are less common due to lack of characterization and lower efficiencies. One such method, supersonic atomization, uses a high frequency electrical current placed over a nozzle made out of a piezoelectric material to induce supersonic vibrations. The liquid stream exiting the nozzle is disintegrated based on the frequency of these vibrations (Masters 1972). This provides a very controlled source of droplets in a narrow size range but is difficult to scale-up for industrial production.

Our research concentrates on the atomization of the feedstock liquid by the carbon dioxide assisted nebulization with a bubble dryer (CAN-BD) process. The method of atomization is a variant of pneumatic atomization, where, instead of a low pressure nebulizing gas, a highly pressurized, near critical gas (generally carbon dioxide) is mixed with the liquid before exiting the nozzle. The nozzle used is exceedingly simple, a small (75-µm or 100-µm) tube made of fused silica or stainless steel, which distinguishes this variant from other forms of high pressure atomization. The high density of the pressurized gas increases the M_{air} in Equation 4, which allows for increased mass flow rates of the stock without significant increase in droplet diameter. The mixture of the atomizing gas and stock is achieved by directly opposing flow in a low volume mixing tee. During this mixing, it is hypothesized that the high pressure gas partially dissolves into the stock solution (up to 6% by mass), which creates a near saturated solution when the droplet is returned to atmospheric pressures as the spray is directed into the drying chamber. The rapid dissolution of the dissolved gases, and evoltion upon decompression, results in the fragmentation of liquid bubbles in the spray, further decreasing the average size of the droplets. Additionally, the rapid expansion of the near critical gas adds frictional forces to the liquid spray, causing additional liquid fragmentation. The benefit of small initial droplets is that less total heat is required to dehydrate the droplets and the final dry particles result in accordingly smaller particles. Atomization using the CAN-BD process has been shown to readily produce droplets which, upon drying, form particles with mean D_A values in the respirable range (5 µm to 1 µm).

1.3.2 Powder Drying

Drying of the atomized feed stock is achieved by exposing the dispersion to a gaseous media of low humidity. The most common drying gasses used are conditioned/heated environmental air or warmed or heated dry nitrogen gas, if an inert environment is desired. Each droplet in a spray is exposed to different local drying conditions due to its differences in the turbulence and flow of the drying gas and the local changes due to the drying of neighboring particles (Cal and Sollohub 2010). Optimization of the drying process generally focuses on the average drying conditions of the largest droplets, which will be the slowest to dry, and represent a larger mass of the final product, which may adversely affect the smaller dry particles upon co-collection. The drying conditions are controlled by drying chamber geometry and drying gas flow rate and heat.

Different drying chamber geometries can result in differences in droplet residence time, air exposure and turbulent mixing. The primary types of drying chambers are concurrent, where the aerosol spray and drying gas flow in the same direction; counter-current, in which the spray and drying gas flow in opposite directions; and mixed flow dryers, where spray flows against and with drying gas in two separate phases. Concurrent flow can result in lower residence time, but droplet-droplet interactions are minimized. Counter-current chambers have larger droplet residence times, but require heavier droplets/low drying gas flow rates to separate the product from the drying gas flow. Mixed flow chambers result in longer residence times and increased drying gas exposure due to turbulent mixing in the drying gas, but also increase the risk of droplet-droplet interaction and aggregation by mixing the drier particles with newly-sprayed wet droplets (Masters 1972).

Drying gas flow rate and temperature can be adjusted more readily than other spray drying parameters and are usually chosen as the primary parameters for drying optimization. These parameters can be monitored by the flow rate and temperature (inlet temperature) of the gas into the chamber and the temperature of the exiting gas (outlet temperature). The flow rate of drying gas determines the residence time of the droplets and the turbulence of the drying gas in the chamber, Equation 7.

$$Re = u\, d_h / v \qquad (7)$$

The Reynolds number (Re) for flow in a pipe is a unit-less number that can be calculated using the velocity of flow (u), hydraulic diameter of the pipe (d_h), or drying chamber, and kinematic viscosity of the fluid (ν), or drying gas. For Re < 2300 flow will be laminar, 2300 < Re < 4000 flow will be transient and Re > 4000 flow is considered turbulent. Particles entrained in a laminar flow can remain separated from neighboring particles and are less likely to aggregate but solvent exchange with the gas may be limited by a boundary layer. Particles entrained in a turbulent flow will be at a greater risk of impacting with other particles and onto the dryer surface, but will have a smaller boundary layer and greater solvent exchange with the drying gas. These conditions are highly variable with the variant of spray drying used and the desired effect.

Inlet and outlet temperature can be controlled to affect the drying rate and final particle properties (Cal and Sollohub 2010). Increased inlet temperatures can increase the evaporative capacity of the dryer and may result in the formation of lower density, hollow or porous particles and particle component inhomogeneity due to the rate of droplet evaporation (Cal and Sollohub 2010). Inlet temperature is controlled by preheating drying gas through heat exchange. Outlet temperature can be controlled by adjusting feed flow rate. By adding additional atomized solution to the drying gas, more of the heat of the drying gas will be absorbed by the liquid in the spray as the heat of vaporization, resulting in a lower outlet temperature. Due to evaporative cooling, particles that still contain solvent at the outlet may be cooler than the surrounding air. Dry particles, however, will have the same temperature as the surrounding air (outlet temperature). The outlet temperature is representative of the highest possible temperature of the particle exiting the drying chamber. This variable needs to be controlled to prevent thermal degradation of the dried material and should be kept as far as possible from the glass transition of the final powder to prevent loss due to powder stickiness (Masters 1972).

The CAN-BD process used in our research uses a 1.5 L glass conical, concurrent drying chamber. Dry nitrogen gas is heated by an in-line thermal transfer element and introduced to the spray at a typical rate of 30-40 L/min (giving an average residence time of 3-2.5 seconds). Some turbulence is noted, as there is seen adhesion of powder uniformly on the internal glass surface of the drying chamber, which may indicate a higher residence time for the drying particles. Inlet air temperature is controlled, by adjusting the voltage supplied to the heating element, to produce outlet temperatures from 50 to 80 °C. Drying under lower outlet temperatures decreases the exposure of the powder and active ingredients to the possibility of thermal degradation.

1.3.2 Particle Collection

After the initially atomized droplets are dried into dry particle form the particles need to be collected through separation from the drying gas. The most common methods of separation are through filtration, inertial separation in a cyclone, or electrostatic separation (Masters 1972). Filtration is the simplest and most efficient form of separation. In filtration collection, particles are transferred from the drying gas to the surface of a filter by passing the drying gas with suspended particles through a filter with pore size smaller than that of the particles. Filters may be porous material due to interlocking fibers, sintered materials or etched cylindrical pores and may be placed as a terminal surface or as a collecting bag (Masters 1972). Material can be collected at intervals by intermittent removal from the filter, or continuously, in the case of back-flow bag filtration, through bursts of counter current air with collection by deposition into a removable reservoir (Vehring, Foss et al. 2007). Separation by cyclone involves sending the particle laden exhaust air in a spiral pattern down and then up a conical cyclone collection device, eventually leaving through the top. Particles with sufficient inertia will impact on the walls of the cyclone device and fall or slide down to the collection chamber below. This method is commonly used due to its simplicity, continuous operation and ease of powder removal. One notable difficulty with this method of separation is that particles with very low inertial (such as particles below 10 μm in D_A) are not efficiently separated using normal flow rates and can remain suspended in the vented exhaust air (Truong, Bhandari et al. 2005). Another effective method of particle removal is electrostatic precipitation, where suspended particles receive a static charge and are removed from the air flow by applying a voltage over the airflow directed towards collection plates.

Dried powders produced by the CAN-BD process are collected on "dead end" membrane filters. Air flow is directed over the surface of a membrane filter where entrained particles are removed. These particles may be exposed to additional heated drying air after the spray has been stopped, through a secondary drying process. Particles are collected either by scraping off the filter at the end of a run or through continuous collection by an engineered dual filter system.

Once powders have been collected it is necessary to store the particles under conditions that will not compromise their activity or physical properties. Our group has used sealed glass screw top vials, plastic vials and various aluminum foil/polymer laminate film materials as high barrier overwrap packages containing packets or "pillows" of molecular sieves or other desiccants. Our primary method of storing bulk powder is in a closed glass screw top vial with Teflon liner, heat-sealed inside an aluminum-based laminate overwrap material with packets of desiccant material, usually molecular sieves. This prevents moisture ingress into the powder and possible degradation due hydrolysis, oxidation or exposure to light. With our collaborators at Aktiv-Dry LLC, we have also designed single dose particle storage containers consisting of mold-formed plastic backing with a recessed slot to hold the powder and covered by a peelable aluminum foil strip heat sealed to the backing. This allows for simple loading of a single dose into the Aktiv-Dry/CU-designed PuffHaler dispersal device for delivery of the dry powder aerosol vaccine.

1.4 Inhaled Powder Product, Dispersal and Delivery

There are many available dry powder inhaler (DPI) devices available on the market, but most of these are “passive”, requiring the patient to inhale forcefully upon command. “Active DPI devices” provide the addition of external forces to disperse the powder as an aerosol for a patient to inhale, while powder in passive devices typically must be dispersed as aerosols by the breathing of the recipient, who must be old enough, and well enough to inhale aggressively. As our primary goal for measles vaccination is infants, who cannot be depended on to breathe on command, we designed an active delivery device, called the PuffHalers.

The major factor in the efficiency of a DPI is its capability of powder dispersal. The bulk stored particles for delivery are in direct contact with each other and require additional external energy to break up the aggregates into individual particles for inhalation. There are many different possible methods of dispersal used with different DPIs, including pressurized air from pumps or contained gas, sonic dispersion and electrostatic dispersion. In order to remove the need for an electrical power source, often an unavailable luxury in underserved areas where vaccination is most needed, our group designed, and tested a hand-powered air bulb for powder dispersal in the PuffHaler. When squeezed, the hand powered bulb releases air at a pressure controlled by the opening of a tetrafoliate pressure-release valve. This pressurized puff of air is sufficient to disperse and aerosolize vaccine powder from the opened powder container placed in the molded plastic PuffHaler device. The powder-laden air inflates an anti-static reservoir bag, with attached mask, which can be placed over the mouth and nose of the recipient for delivery to the lungs by at liberty breathing.

The PuffHaler device has been shown to deliver our powders effectively to the lungs of cotton rats (Masters 1972) and rhesus macaques (Hinds 1999) through at-liberty breathing. The device was also designed with reusable components and inexpensively produced disposable components, resulting in a total cost per delivered dose of ~ $0.10, for the delivery system, which is comparable to the cost of vaccine delivery by injection with needle and syringe. The cost of the stabilizing sugar, *myo*-inositol, used in the inhalable dry powder measles aerosol vaccine to replace the sorbitol used in the present commercially available lyophilized measles vaccine, is not expected to add significantly to raw materials costs, so the new vaccine with the PuffHaler, or with BD-Technologies' Solovent DPI, following human clinical trials, should be comparable in price to the EZ measles vaccine product sold by the Serum Institute of India in 127 countries throughout the world.

1.5 Research Objectives

Through the design and production of an inhalable dry powder measles vaccine we have developed a safe and effective alternative measles vaccination to traditional delivery by injection. In the design of this final product, extensive research was conducted on optimizing production parameters and formulation. Characterization was conducted on the feed material and resulting powder, throughout subsequent packaging and during stability tests. This characterization revealed variances in placebo material and helped to better understand the stability of the physical and chemical properties of the vaccine powder.

A powder was designed and produced to study the toxicity of a novel inhalable excipient, *myo*-inositol. The processing and characterization of pure myo-inositol powders revealed an anhydrous metastable crystal polymorph of *myo*-inositol which was formed under specific process variables. Due to its metastable nature this polymorph is to be avoided in particle production but this method of producing a relatively pure form of a unique polymorph may provide additional insight into the chemical properties of *myo*-inositol.

In order to provide an alternative to the currently commercially available liquid HPV vaccine, a dry powder protein subunit Human Papilloma Virus vaccine was designed and produced. This dry powder vaccine shows no evidence of protein degradation during processing and retains the HPV16 L1 protein conformation required for antigenic activity, as determined by ELISA testing. This powder has been delivered to Sprague Dawley rats by insufflation, reconstitution with alum adjuvant and injection and sublingual pellet with no adverse reactions. Immunogenicity results are forthcoming, but not available at the time of the writing of this dissertation.

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Chapter II Methods

2.1 Powder Preparation and Processing

All powders were produced using the Carbon Dioxide Assisted Nebulization with a Bubble Dryer (CAN-BD) process. All vaccine powders were prepared inside a LABCONCO Purifier Class II Biosafety Containment Cabinet Delta Series using a Bubble Dryer Model #BD-1000, Temco Instruments, Tulsa OK for liquid and gas control and monitoring. Pump and temperature data were recorded using the Temco Instruments Smartdry -2© v1.01 program. Carbon dioxide flow rates, density and temperature were monitored by a Micro Motion Mass Flow Meter and recorded by the Prolink II v2.6 Software. Standard operating parameters were as follows. CO_2 pressure was set to 1200 psi, and CO_2 pumps were temperature controlled (25°C to 30 °C) by a Thermo Electron Corporation Neslab RTE 7 heat exchanger circulating ethylene glycol. The aqueous solution or suspension flow rate was set to 0.3 mL/min to 2 mL/min. Solutions were prepared from 1 % to 20 % (total dissolved solute by mass) with 18 MΩ water from a Barnstead Nanopure Diamond water purifier. The Nitrogen drying gas flow rate was set at 30-40 L/min through the Pyrex glass drying chamber and heated to 40-80 °C. The regulator pressure was set to 30 to 60 psi. The solution of interest was nebulized through a 10 cm long, 75 μm ID silica restrictor (Polymicro Technologies LLC Part # 2000019). For powder production runs of 2 g or less, the dry product was collected on a dead end filter (MAGNA, Nylon, Supported, Plain 0.45 Micron 142 mm Diameter, GE Water and Process Technologies) inside a stainless steel filter assembly. Powder was collected from the filter using a stainless steel spatula after the run. Larger processing runs were collected on a dual filter, back-pulse system designed by David Krank at Aktiv-Dry. In the dual filter back pulse-system, the drying chamber exit gas flow was directed through a 90 degree glass elbow; some additional loss was noted due to particle inertial impaction in the area of this change in flow. The dual filter back-pulse assembly was observed to have lower temperatures (30 to 40 °C) than the outlet temperature exiting the drying chamber (60 °C). The filters used in the dual back pulse system were MAGNA, Nylon, Supported, Plain 0.45 Micron 142 mm Diameter, GE Water and Process Technologies. When using the dual filter back pulse system, powder was periodically cleared from the primary filter (top) and dropped to the collection filter (bottom) using 5 bursts of 15 psi dry nitrogen every 10 minutes. These pulses transferred dry solid collected on the top filter to the bottom filter, where additional drying gas was passed through the powder at 15 L/min to provide additional drying (moisture content for the measles vaccine was usually 0.5% to 2% water by mass using this method). Powders were collected directly from the dual filter back pulse system into a sealable glass jar.

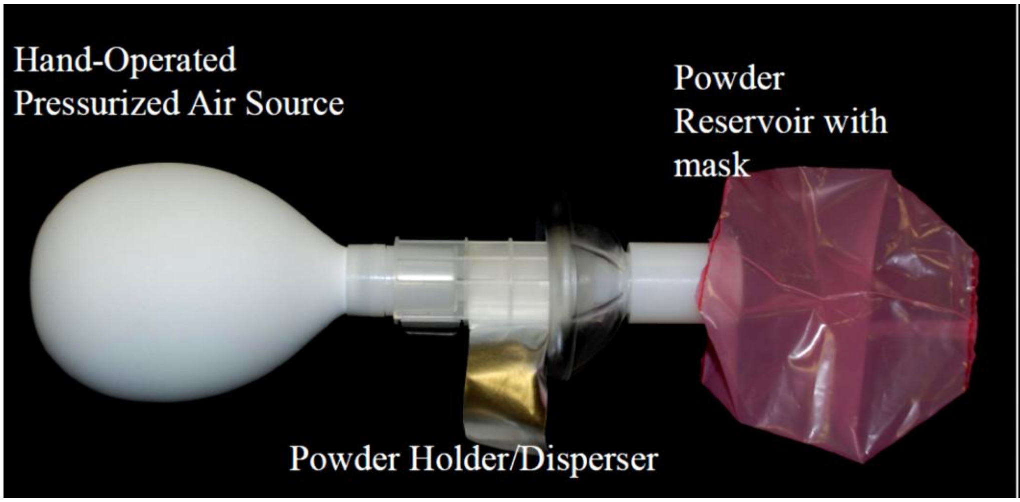
Powders were stored in clean glass or plastic vials stored in high barrier aluminum foil-polymer laminate overwrap with molecular sieve desiccant pillows (Desiccare Inc. 0.5 g Molecular Sieve Pak). Temperature sensitive powders were stored in a refrigerator 2 to 8 °C. Temperature was monitored continuously using Thermocron or Hydrochron iButtons. Materials with active ingredients (measles vaccine, protein or unstable crystal structure) were stored strictly at 2-8 °C, to prevent degradation. Placebos and non-active powders could be stored at room temperature without any observed detrimental effects.

2.2 Andersen Cascade Impaction

There are a variety of methods available for the analysis of geometric particle sizesincluding analysis of electron microscopy images, light scattering and sieving methods. Geometric particle size may not be the best representative variable when analyzing the dynamics of inhaled particles. A better measure of the potential particle performance is the aerodynamic diameter, which separates particles based on their behavior when entrained in a laminar flow, as compared to a model particle that is perfectly spherical with a unit density of 1 g/cm^3. A variety of methods are available to assess the aerodynamic diameter of particles using time-of-flight or inertial impaction in to a liquid (impingers) or onto a series of plates (cascade impacters). As our powder was intended for the delivery of a live-attenuated virus in a complex formula, we required an analytical method that was portable enough to fit in the biosafety containment cabinet, easy to clean thoroughly and did not require extensive chemical analysis of new formulation constituents. Andersen Cascade Impaction is a common method of aerodynamic diameter determination of inhaled pharmaceuticals and is specified in the US Pharmacopoeia. Most of the particle size analysis performed by our group was conducted using a four- or eight- stage Andersen Cascade Impactor with gravimetric analysis.

2.2.1 Four-Stage Andersen Cascade Impaction

Particle size in the respirable range was observed using the modified Westech 8-stage Non-Viable Cascade impactor (Andersen Cascade Impactor or ACI). All weighing of hygroscopic materials was conducted a dry environment controlled to have a RH <15%. Before assembly, a pre-weighed glass fiber filter (Pall Life Sciences A/E Glass 81 mm) was placed in the F stage and held in place by a silicone polymer ring. Another pre-weighed filter was placed on a plate with its lip oriented upwards, the plate was placed on top of stage F. The stages F, 3, 1, and 0 and the neck of the instrument were assembled on top of the base and a solid plate was placed between stages 3 and 1 and a plate with a central hole was placed between stage 0 and stage 1. The springs were set to seal the system and the base was connected to a vacuum pump (Figure 2.1). If the RH of the room was above 15%, the tests were conducted inside a dry box dried to no more than 15% RH with dry nitrogen. In common operation, an Aerolizer® Inhaler Demonstration Unit (Schering Corporation) and a gelatin capsule were used for powder dispersal. The gelatin capsule was filled with 8-12 mg of the powder of interest; filling was conducted in a dry box when the room humidity was above 15%. The capsule was shocked five times over five seconds using a “static gun”, which helped to discharge static charge build up in the powder which was then placed in the Aerolizer. The Aerolizer capsule puncture needles were compressed to puncture the capsule ends and the Aerolizer was then closed. The vacuum pump attached to the ACI was turned on and a flow meter was used to ensure that the flow rate was 28.3 standard liters/minute, as specified by the USP and manufacturer protocol. Previous tests ensured that the pressure drop of the ACI was at least 4 Kpa. The Aerolizer was placed in the ACI neck inlet and the connection was sealed by a rubber stopper. While the Aerolizer was in contact with the ACI, it was ensured that the capsule was rattling inside; if the ratting stopped, the capsule was reseated and then returned to testing. After pulling air through the Aerolizer for eight seconds, the Aerolizer was removed and the vacuum pump was shut off. In some cases the BD Solovent or Aktiv-Dry PuffHaler^{TM} (Figure 2.2.1) were used for powder dispersal. In these cases, prefilled capsules or foil packs were actuated using the proper dispersing method and the powder bolus was either introduced directly to the inlet of the ACI or through the evacuation of the PuffHaler aerosol reservoir bag (made of an antistatic polymer material) or the Solovent spacer box.



2.2.1 Figure- PuffHaler and component parts

The empty capsule, the filter in stage F and the filter under stage 3 were weighed. The mass of powder on the stage F filter (M_F) was the mass below 3.3 μm in aerodynamic diameter and the sum of the masses on stage F filter (M_F) and Stage 3 filter (M_3), compared to the mass of powder loaded into the device (M_L), gave the mass below 5.8 μm in aerodynamic diameter. Powder was characterized by %< 3.3 μm by Equation 2.1 and % < 5.8 μm by Equation 2.2. The value of % < 5.8 μm was considered the respirable (inhalable) fraction.

$$\frac{M_F}{M_L} * 100\% = \% < 3.3\, \mu m\ D_A \qquad (2.1)$$

$$\frac{M_3+M_F}{M_L} * 100\% = \% < 5.8\, \mu m\ D_A \qquad (2.2)$$

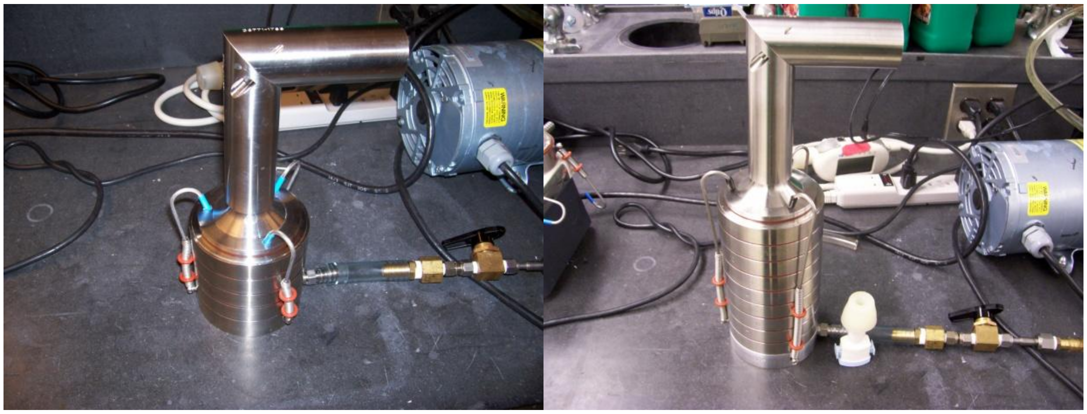


Figure 2.1 Four (left) and eight (right)-stage Andersen Cascade Impactors

2.2.1 Eight-Stage Andersen Cascade Impaction

In order to analyze the powder using a larger number of size bins, the 8-stage Andersen Cascade Impactor assembly was used. The United States Pharmacopoeia (USP) method 601 was followed. This was conducted in much the same way as the 4-stage device arrangement using the ACI with stages F, 8 through 0. Gravimetry was used with this method. Additional chemical methods were occasionally used to improve sensitivity and validate the analysis. As only a maximum of 1 or 2 mg of powder were expected to deposit on the metal stages of the ACI, a much more sensitive technique, Total Organic Carbon (TOC) analysis, was employed instead of gravimetric analysis when necessary (Masters 1972).

The procedure laid out by Rebits et al called for an extensive cleaning procedure to remove as much organic carbon as possible with ultrapure water. All materials were allowed to dry and were assembled on clean aluminum foil. The plates were coated with a thin layer of Dow-Corning silicone grease and the ACI was assembled. After the powder was aerosolized into the device according to the previously mentioned gravimetric method, the device was carefully disassembled and rinsed with ultrapure water. The water samples were transferred to clean TOC vials and tested for TOC and Inorganic carbon using a SIEVERS® Model 800

Total Organic Carbon Analyzer with a SIEVERS® Model 820 Automatic Sampler (both from GE Analytical Instruments, Boulder, CO).

2.3 Emitted Dose Device

In order to obtain emitted dose data for individual powders using a designated Dry Powder Inhaler (DPI), the amount of powder deposited on the filter from dispersing into a standard emitted dose device was determined. A 46-mm glass fiber filter was weighed and placed in a USP emitted dose device. The device was connected to a vacuum pump and air was pulled at a flow rate of 28.3 Standard L/min. The DPI of interest was actuated to disperse the aerosol into the opening of the emitted dose device. The weight of the powder on the filter with deposited powder was divided by the loaded mass to determine the percent emitted dose.

2.4 Model DP-4 Dry Powder Insufflator (PennCentury)

The dry powder insufflator for rat studies was tested using the ACI and the Emitted Dose Device. The powder reservoir of the insufflator was filled with 5-10 mg of powder and sealed. The mass of the powder added was determined be the difference in the weight of the empty insufflator and the filled insufflator. A 3-ml syringe was used to dispense 0.5-2 ml of air to aerosolize the powder into the mouth of the running ACI or Emitted dose device. For each actuation, only 2-3 mg of powder was aerosolized. In order to get enough powder to reliably determine the particle size or emitted dose, several (3-5) actuations were conducted and the final result was the average of these replicates. Testing of powder dispersed using the insufflator may not be fully represented by the ACI results, as additional momentum is supplied to the powder through the dispersal air. A significant amount of powder was noted to have impacted on the angle change at the end of the neck portion of the ACI. In general, dispersion of powder using the Insufflator device resulted in observed FPF values equal to or greater than those observed for the same powders using the Aerolizer as a dispersal device.

2.5 Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) analysis on powders and dry bulk material was conducted using a Perkin Elmer Diamond DSC. Samples (1-10 mg) were loaded into aluminum DSC sample containers in a dry environment (15% RH) and hermetically sealed. Sample containers were placed in the sample pan of the DSC with an empty sample container placed in the reference pan. The DSC was controlled by Pyris software. Samples were generally analyzed using a heating rate of 100 °C/min to maximize signal. The samples were cooled to –20 °C, or well below the potential zone of interest for the material. Samples were heated beyond the area of interest at 100 °C/min and then cooled at 100 °C/min to the starting temperature and allowed to equilibrate for 2 minutes. The sample was then heated a second time past the temperature zone of interest at 100 °C/min.

The data from the first scan was recorded as the thermal profile of the processed material, while the second scan was recorded as the thermal profile of the bulk material, assuming the initial scan was not chemically destructive to the sample. In the case of a powder, the first scan is the thermal profile of the individual particles in aggregate as they go through the glass transition or melting point and lose their physical powder properties and spread out over the bottom of the sample container. The second scan represents the thermal profile of the bulk material spread out over the bottom of the capsule. In general, the glass transitions observed in first scans are at lower temperatures than the glass transitions of second scan and first scans generally have larger exothermic overshoots than the second scans. This represents the thermal properties and artifacts of the particles vs. the bulk; particles have much larger surface area and are more affected by the heat transfer. The larger exothermic overshoot is due to the energy associated with the particle melting and fusion and is not as pronounced in the bulk.

Glass transitions were identified by S-shaped slight inflections of the heating curve and were analyzed by Pyris software. Tg Onset (°C), Half Cp extrapolated (°C) and ΔCp (J/g °C) values were reported and used for powder comparisons. Melting points were identified by large exothermic peaks in the heating curve. Melting points were analyzed by the Pyris software and Onset, midpoint and peak height or area were recorded. Crystallization events were observed as exothermic events on the heating or cooling curve and analyzed by the Pyris software. The cooling curve data was not used for analysis as the instrument did not regulate the cooling stage in an analytically reliable manner .

2.6 Karl Fischer Titration Moisture Analysis

Powder and bulk solid moisture content was determined by Karl Fischer moisture analysis. Powder or bulk solid (10-100 mg) was transferred into clean/dry 1 dram vials in a dry environment (no greater than 15% RH). About 1-2 ml of dry methanol (at most 0.3% water by mass) was added to the powder in the vials. The vials were sealed, mixed thoroughly on a vortex and centrifuged to remove the suspended powder to a pellet. The humidified methanol was removed through a syringe and injected into a Denver Instruments Model 260/275 KF Coulometric Karl Fischer Titrator using Riedel-de Haen Hydranal-Coulomat AG and Hydranal Coulomat CG Catholyte. Moisture content was reported and compared to the injected methanol mass and powder mass to determine the total moisture content of original powder.

2.7 Powder X-ray Diffraction

Powder X-ray diffraction (XRD) was conducted on the powders to determine crystallinity, compare with literature XRD patterns and identify significant peaks. Powder or bulk solid material was placed in a thin layer in an acrylic sample cuvette. The sample cuvette was placed into the rotating sample holder of a SCINTAG PAD-5 XRD instrument and analyzed from 2 Theta= 5° to 45° at a scan rate of 2°/sec. The Instrument used a Cu K-alpha source set to 1.540562 Å. The powder was determined to be amorphous if no clearly visible peaks appeared above the acrylic holder background or an amorphous hump was observed. A sample was determined to have some crystalline characteristics when visible peaks were observed. Peaks were identified and evaluated by comparison with an International Center for Diffraction Data (ICDD) database.

2.7 TSI Aerosizer

In the initial stage of this research, the TSI Aerosizer was used to assess the aerodynamic particle size range. Powder (about 10 mg) was placed in the sample cup of the TSI Aerolizer 3225 with a 3230 Aero-Disperser and the sample run was controlled by the standard TSI software. Powerful bursts of air were used to fully aerosolize and disperse the powder. Powder sizes were determined by time-of-flight between two light sources. The data produced by this instrument represented the ideal particle size range of the powder, different values are expected for less powerful dispersion methods such as ACI with the Aerolizer, PuffHaler and Solovent due to particle cohesion and adhesion from static forces, stickiness and Van der Walls forces.

2.8 Scanning Electron Microscopy (SEM)

Early SEM pictures were taken at the Laboratory for Environmental and Geological Studies in the CU Geology Department. Powders were taken to the microscopy facility in a sealed container over molecular sieves on conductive tape on aluminum SEM stubs. Care was taken to spread the powder into a thin layer to allow the electrons to pass through the sample to the tape and minimize sample charging. The powders were gold sputter coated at the Geology facility and placed in the JEOL ISI SX30. Pictures were taken at 5-30 KeV and recorded on film. Pictures were later converted to digital images with a scanner and processed by Photoshop software.

Later SEM pictures were taken with a low vacuum SEM (LVSEM), JEOL-6480LV, at the Nano Characterization Facility in the CU Discovery Learning Center. A thin layer of sample was spread onto conductive tape on an SEM stub in a low humidity environment (less than 15% RH) and the samples were transferred in a dry desiccator to the facility. Samples were placed into the LVSEM sample chamber, a vacuum was applied to reduce the pressure to 1-10 Pa, and images were captured using 5-30 keV accelerating voltage. This instrument was designed to image materials that are prone to charging and well-resolved images were obtained without gold coating of the particles and materials. If particles were observed to melt under the electron beam or minor charging was noted, the voltage of the instrument was reduced. Highly charged aggregates of particles were avoided in favor of well-dispersed particles in direct contact with the conductive tape. If excessive charging was observed (i.e., moving particles, intense or changing contrast) a new image was obtained using gold-coated particles. Digital copies of the pictures were saved and processed by Photoshop software.

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Chapter III Characterization of a Dry Powder Measles Vaccine

3.1 History of Measles

Measles, originally known as “Rubeola” in the middle ages, is an RNA virus of the Morbillivirus and Paramyxoviridae family that has been documented in humans since at least the seventh century (Masters 1972). Infection with the measles virus begins with symptoms such as cough and fever followed by a maculopapular rash and small red-rimmed white dots (Kolik’s spots) on the mucous membrane of the oral mucosa. Measles may result in diarrhea, pneumonia or encephalitis in children, with greater severity and danger in younger children. Measles is an incredibly contagious disease with an estimated infection rate of at least 95% (Kisich, Higgins et al. 2011) and was once considered an unavoidable childhood ailment. A 2004 WHO study estimated that, globally, 424,000 deaths were caused by measles directly or indirectly that year, most of these deaths occurred in the low and middle income populations of Africa and South East Asia (Lin, Griffin et al. 2011). This study also estimates that, despite the existence of an effective vaccine, measles directly causes up to 4% of all deaths in children under 5 years of age. This puts measles as the leading vaccine preventable childhood disease and shows the disproportionate burden that it puts on the developing world. In order to reduce childhood mortality and morbidity in the developing world, reduce the risk of massive measles outbreaks and in hopes of fully eradicating the measles virus, new and efficacious advances in measles vaccination need to be developed and implemented.

The first reported experimental measles vaccination was performed in 1758 by Scottish Doctor Francis Home, who gathered blood from measles patients “when it contained the morbilic matter in the highest state of acrimony”, or at the peak of fever, when the patient’s natural defenses were working hardest against the virus (Rebits, Bennett et al.

2007). Delivery of this blood, or nasal discharges from measles patients, to uninfected children reportedly proved to provide protection from measles infection. Unfortunately subsequent research did not back up these initial observations and extensive research into the development of a measles vaccine was not resumed until the 1950s-1960s. In 1954, researchers Enders and Peebles were the first to isolate a strain of measles, referred to as the Edmonston strain, named for David Edmonston, a 13-year old boy from which it was collected (Artenstein 2010). Research into methods of viral attenuation began in the early 60s and these attenuated viral bodies were incorporated into vaccines which proved to provide good immunogenic response (Black 1982). Viral infectivity was reduced by multiple passages through non-human cell lines. Many different methods of viral attenuation and strains of measles have been used for vaccination since the 1960s. Currently, the most extensively used live attenuated measles vaccine strain in developing countries is the Edmonston-Zagreb (EZ) strain, which is attenuated by passage in human diploid cells (Mathers, Fat et al. 2008). It is this form of attenuated measles virus, obtained from the Serum Institute of India, in Pune, that we have reformulated for dry powder delivery.

3.1.1 Measles Vaccination

Literature reviews of measles vaccination field campaigns have shown vaccine effectiveness of 77.0% when received at 9-11 months and 92.0% when delivered to children ≥ 12 months (Enders 1961). The difference in effectiveness for these age groups is due to the presence of varying levels of maternal antibodies in younger children. If the attenuated virus is prevented from replicating by existent antibodies, the immune system of the recipient does not develop a strong immunity to the infective agent. This study also showed that the average vaccine effectiveness for 2 doses of a vaccine against measles versus no vaccine at all was 94.1%, showing excellent protection from measles with a 2-dose vaccination campaign. Measles vaccine is considered to be the only candidate for successful elimination of measles from defined geographical locations. In 127 developing countries, the strain of measles vaccine provided by the Serum Institute of India is the Edmonston-Zygreb (E.Z strain). In the U.S. and Europe the primary measles vaccine used is the Attenuvax , an additionally attenuated strain of the Enders' attenuated Edmonston strain, produced by Merck, or Priorex from GlaxoSmithKline, using the Schwartz attenuated measles strain.

Extensive field studies show that measles can be essentially eradicated with thorough vaccination campaigns. In the African country of the Gambia, elaborate vaccine campaigns in the late 1960s reached an estimated 96% of children between 6 and 36 months and reduced the incidence of reported measles cases from the thousands to the single digits, or essentially non-existent (Artenstein 2010). Vaccinations were delivered using a jet-injection gun rather than disposable needles and were maintained with regional vaccination campaigns every 12 months, where all children between 6 to 18 months old were vaccinated. This maintained low rates of measles infection, until funding ran out in 1971. In 1977-1979 an average number of 2,036 cases of measles were reported in the Gambia (approximate population 400,000) (Black 1982). If a concerted effort is to be put forward to eradicate this disease through vaccination campaigns in wide areas there is a need for future economical, effective and safely delivered method of measles vaccination. Needle-free options are expected to include wet mist aerosol and the dry powder aerosol (MVDP) that is the subject of this chapter.

In the early 1960s, shortly after the initial formulation of the measles vaccine (using both killed and live-attenuated virus), research was conducted on the immune response to vaccine delivered by inhaled aerosol in Japan, the US and the USSR (reviewed in (Artenstein 2010)). Results of these tests, which used antiquated testing methods and inefficient liquid nebulizers, nonetheless provided evidence that children immunized by the inhalation route could demonstrate seroconversion rates comparable to children given the vaccine by injection. Unfortunately, these successful results were not immediately followed by extensive research into measles immunization by inhalation. The push for additional research into aerosol delivery of vaccines was led by Dr. Albert B. Sabin with his research on and vaccination campaigns employing nebulized liquid vaccine, conducted in the early 1980s.

In 1982 and 1983 field studies conducted by A.B. Sabin et al in Mexico and Brazil showed that children can be effectively vaccinated for measles with mass vaccination campaigns using the aerosol delivery route (Uzicanin and Zimmerman 2011). In these campaigns, vaccination was conducted using lyophilized measles vaccine originally intended for injection, rehydrated with sterile water directly before aerosol delivery. The liquid vaccine was stored on ice and nebulized using an industrial air pump and delivered to children using inexpensive and simple face masks (modified "Dixie cups"). Dr. Sabin estimated in 1991 that, using a foot powered nebulizer pump and reconstituted lyophilized measles vaccine, an organized campaign following Sabin's procedures could successfully vaccinate over 10 million children by wet mist in one day for a cost of $300,000. This was $1/6^{th}$ of the cost of a similarly sized 30 day vaccination campaign using disposable needles (Sabin 1991). Interestingly, Sabin's hypothesis is mirrored by a recent paper analyzing the cost-effectiveness of the most promising innovations in measles vaccination (Sabin 1991). The results of the study show that, despite the large cost of initial research and development, aggregate cost savings over the next 40 years could be as large as $154.1 million for a nebulized aerosol vaccine and $689.4 million for the inhalable dry powder measles vaccine, being studied here.

3.2 Design of a Dry Powder Measles Vaccine

Our work, under the FNIH Grand Challenges in World Health Initiative, has concentrated on the formulation, analysis and testing of a dry powder live-virus measles vaccine for pulmonary delivery. Using the Edmonston-Zagreb attenuated virus vaccine provided by the Serum Institute of India (Pune, India) a dry powder vaccine, with particles in the inhalable range (1-5 μm D_A) for delivery to the lung has been formulated and processed by CAN-BD (Sabin 1983). The primary change made to the powder formulation, as compared to its initial lyophilized form, was the replacement of the sugar sorbitol (at a level of about 50%(m/m) in the final powder) with an equivalent percentage of the novel excipient *myo*-inostiol. This *myo*-inositol containing formulation demonstrated a significantly decreased hygroscopicity and a higher glass transition temperature than the sorbitol-based commercial lyophilized cake. This shows an increase in the stability of the physical properties, which is required for an inhaled product, where aerosol behavior is intimately related to efficacy. Further tests of our formulation have shown viral stability exemplified by less than 1 log loss of Plaque Forming Units (PFU) under storage conditions of 37 °C for 1 week, matching the stability of the original lyophilized formulation and meeting the WHO requirements of vaccine stability at elevated temperatures. Continued investigation of our formulation has shown less than 1 log loss of activity by PFU after storage at 2-8 °C for 43 months (Sabin, Decastro et al. 1982; Sabin 1991). Data obtained by collaborators at the Serum Institute of India indicate that the reformulated vaccine has a shelf life of 6 months at a storage temperature of 25° C.

Delivery of our dry powder measles vaccine to cotton rats by inhalation shows deposition and replication of measles virus in the lungs, verified by measles-specific reverse transcription polymerase chain reaction (RT-PCR) (Sabin 1991). Measles virus replication was confined to the lungs and airways and showed no evidence of transportation to the brain, liver, spleen or kidney. The powder was delivered to cotton rats using the Aktiv-Dry PuffHaler, where powder was dispersed into a powder reservoir and rats were allowed to breathe at liberty from the suspended powder. An estimated 0.1% of the powder was deposited in the lungs, with some additional deposition of powder in the nasal passages. Despite this low dose, a detectable immune response was recorded in response to the viral replication.

In 2006, a study using a dry powder measles vaccine, formulated by a different research group (Garrison, Bauch et al. 2011), was delivered to a non-human primate model, cynomolgus macaques, by direct insufflation into the lungs (Burger, Cape et al. 2008). While this study showed poor immunogenicity, delivery of our dry powder measles vaccine formulation directly to the lungs by at liberty breathing has shown very positive immunogenic results in non-human primates (Stephen P Cape 2011). Vaccine powder was delivered to rhesus macaques using two different active dry powder inhalers (PuffHaler and the BD Solovent) and direct intranasal dispersion of the powder using the dispersing units of the PuffHaler and Solovent. No adverse effects were noted in the vaccinated animals. All animals showed evidence of viral replication in the cells of the respiratory system and strong anti-measles humoral and cellular immune responses. When challenged with wild-type measles virus 14-16 months after vaccination, all the animals vaccinated by dry powder measles vaccine with a passive dry powder inhaler through a face mask showed complete protection (Kisich, Higgins et al. 2011). These tests validate the efficacy of our dry powder measles vaccine formulation and delivery method and help justify progression of the vaccine to Phase I safety trials in humans.

3.3 Characterization of Dry Powder Measles Vaccine

During the development of our dry powder measles vaccine (MVDP) many tests were conducted to ensure uniform powder and vaccine quality. Powder was analyzed by ACI for particle sizes, Karl Fischer titration for moisture content and differential scanning calorimetry for thermal properties (glass transition (Tg) and melting point). Powder was analyzed for crystallinity by powder X-ray Diffraction (XRD) and for identity by NMR.
All placebo (not containing live-attenuated virus) MVDP powder analyzed showed evidence of being amorphous by pXRD. Powder X-ray Diffractograms, obtained by the procedure outlined in the Methods chapter, showed an “amorphous hump” associated with amorphous

character rather than discrete peaks which would be produced by a significant amount of repeating crystalline cells, Figure 3.1.

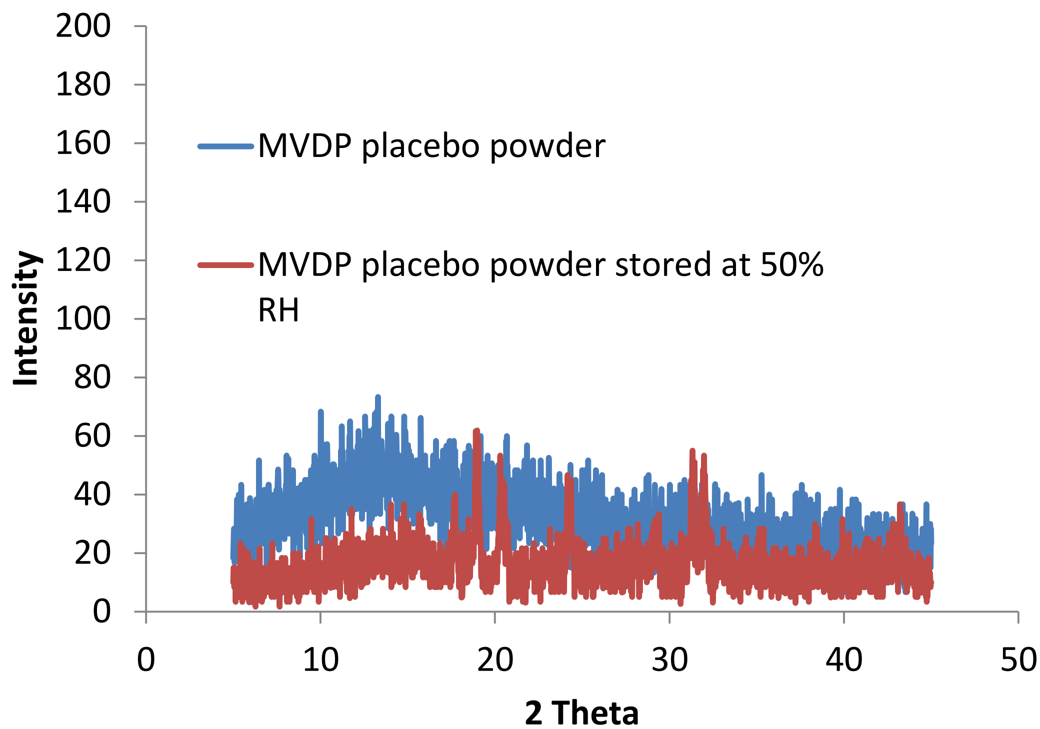

Figure 3.1 pXRD Diffractogram of MVDP placebo powder

Figure 3.1 also shows the pXRD Diffraction pattern for a sample of the placebo exposed to a relative humidity of 50 %. Some sugars are known to transition from a glassy matrix to a crystalline form after exposure to environmental moisture (LiCalsi, Maniaci et al. 2001). It can be seen that no distinct peaks arose in the material exposed to a high humidity. Any apparent differences from the dry placebo are likely due to slight variances in the analysis, such as the sample backing, or due to sample presentation; the moist placebo was in a sticky paste as compared to the fine powder of the dry material.

Due to the limited sensitivity of the pXRD, we cannot discard the possibility of a microcrystalline structure of components of powder. Many of the excipients included in the MVDP formulation will form a crystalline powder when processed by CAN-BD alone or at high concentrations. In combination with the other excipients, such as the plasticizer, gelatin, it is thought that the combined formulation will not contain a high enough local concentration to facilitate extensive crystallization. This produces a powder made of an amorphous sugar glass with an observed Tg of about 60 °C (de Swart, LiCalsi et al. 2007). An amorphous formulation was desired to enhance the stability of the measles virus particles, as crystalline structures are known to have a unyielding character that may lead to the puncture of the viral membrane or the denaturing of the viral proteins (Lin, Griffin et al. 2011).

3.3.1 NMR Analysis of Measles Vaccine Placebo

The raw vaccine material we used in powder processing was prepared in India by our collaborators, the Serum Institute of India (Pune, India), and shipped to the US as a frozen liquid. It was very important to note any differences in the quality and physical properties of this vaccine to ensure the production of a uniform dry powder vaccine. One particular batch of liquid stock material of MVDP sent from India was noted as being less viscous than previous batches of vaccine material. Analysis of total dissolved solids showed a slight decrease in the solids content of the material. NMR identification studies were conducted to determine the cause of the compositional differences between the two liquid powder batches with differing viscosities.

In order to analyze the apparent differences between the original and less viscous placebo three samples were prepared for NMR analysis. Samples of the less viscous liquid placebo and the original liquid placebo were diluted 1:1 with D_2O, in order to reduce the O-H interference in the NMR spectra due to water. A sample of powder prepared from the new placebo was reconstituted to a concentration of 10% total dissolved solids with a 1:1 solution of water and D_2O, in order to be comparable to the liquid placebo. These samples were analyzed to obtain their H^1 NMR spectra by the Varian Inova 500 NMR instrument at the CU NMR facility as described in the Methods chapter. WET water suppression was used in each run and the final NMR spectra were analyzed by MestReNova NMR spectra-processing software.

The NMR spectrum of each sample appeared to have the same constituents, based on brief inspection of the spectra, Figures 3.2.1 and 3.2.2. All of the spectra show the characteristic *myo*-inositol triplet at 4.02 ppm (Lin, Griffin et al. 2011) and all have analogous peaks for the other components. The proton NMR peaks were assigned to different hydrogen environments based on NMR shift tables (Schebor, Mazzobre et al. 2010). The peaks at 7.8 and 7.05 ppm are likely due to an aromatic proton (Ar-H), most likely of histidine. The peak at 0.9 ppm is likely R-CH_3, 1.45 ppm is R_2-CH_2, 1.65 ppm is R_3-CH and 1.85 ppm is C=C-CH_3. These peaks are not present in the NMR spectra of stock *myo*-inositol and are thus associated with the other components (amino acids, gelatin, trace sugars, etc.). The peak associated with *myo*-inositol is at the triplet at 4.02 ppm and is representative of H-C-OH.

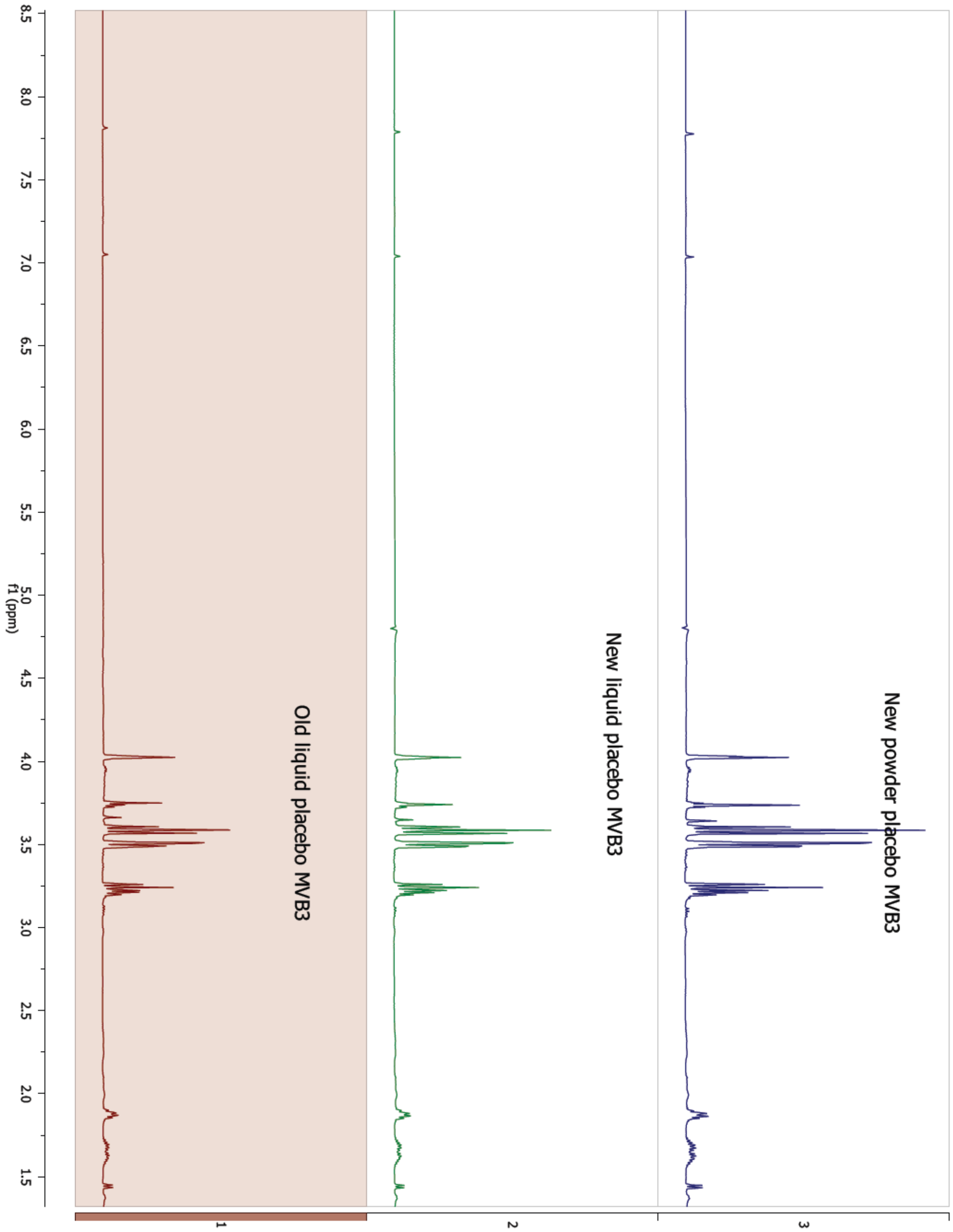

Figure 3.2.1 NMR of measles vaccine placebo

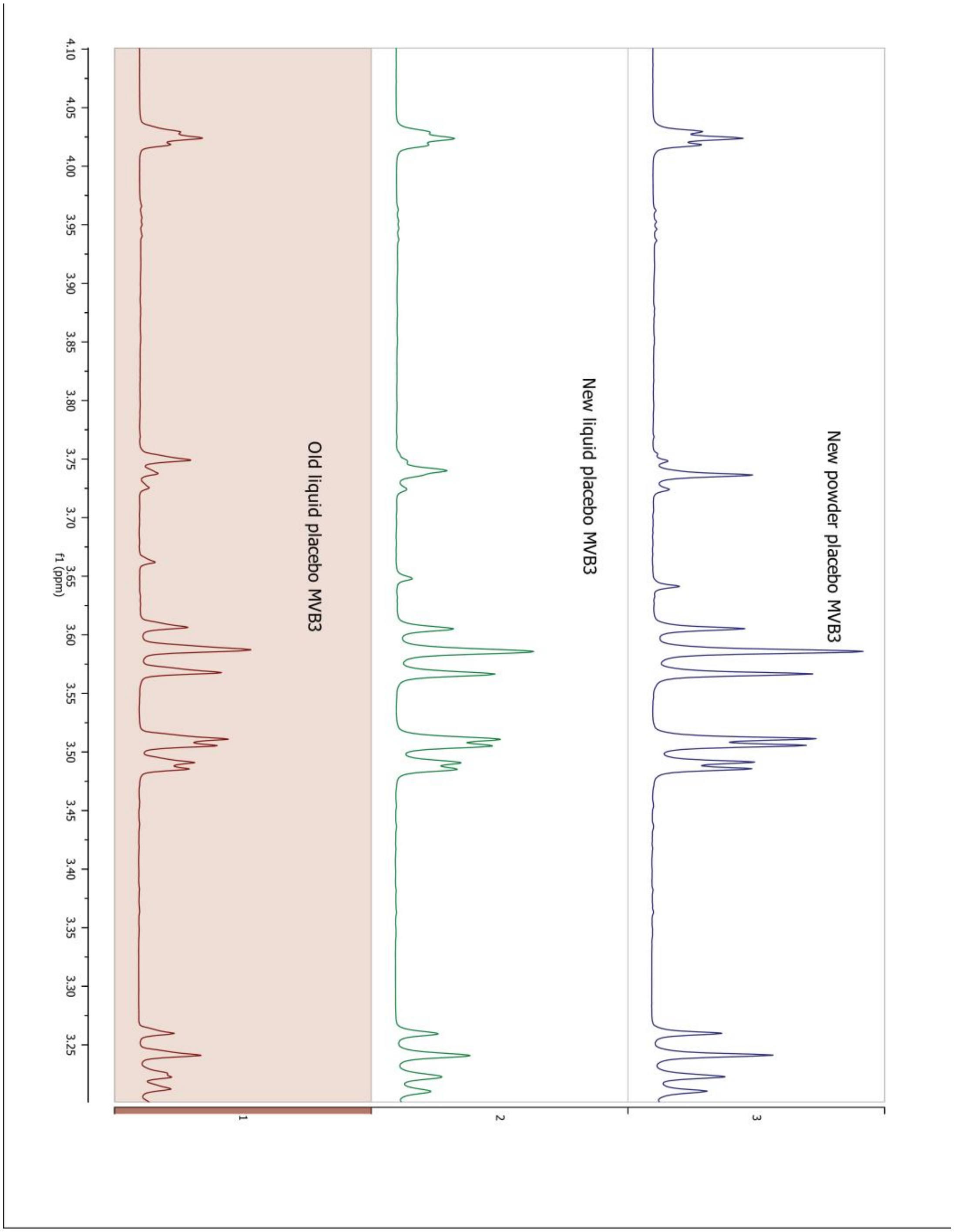

Figure 3.3.1 NMR of different placebos, zoomed into *myo*-inositol region

In order to compare the molecular ratios of the different components, the *myo*-inositol triplet at 4.02 ppm was set as the reference by setting its integrated area to 1.0.

Molecular ratios in a complex mixture were be determined by the relative ratios of the integrated areas of the peaks. For comparison the peaks at 7.05, 7.82, 1.9, 1.65, 1.45 and 0.9 ppm were chosen. The peaks at these values were integrated and the resulting areas were compared between the less viscous placebo liquid, placebo powder made from this liquid and the original placebo liquid spectra (Table 3.1).

***myo* ref 4ppm =1.0**						
Associated protons	C=C-CH_3	R_3-CH	R_2-CH_2	R-CH_3	Ar-H	Ar-H
NMR	1.9 ppm	1.65ppm	1.45ppm	0.9ppm	7.05ppm	7.82ppm
Less viscous powder	0.73	0.82	0.17	0.31	0.06	0.06
Less viscous liquid	0.74	0.8	0.17	0.32	0.06	0.06
average	0.735	0.81	0.17	0.315	0.06	0.06
Original placebo liquid	0.63	0.69	0.13	0.24	0.04	0.04
% difference	14.3	14.8	23.5	23.8	33.3	33.3

Table 3.1 NMR relative integration studies

From Table 3.1 it can be seen that the molecular ratios between the less viscous placebo liquid and powder samples are nearly identical, but when compared to the original liquid placebo, the molecular ratios are consistently lower. This implies that the ratios of *myo*-inositol to the other components of the placebo mixture are the same before and after CAN-BD processing, but are different between the new and old placebo formulation. Furthermore, the changes in ratios are not consistent, as would be expected if the concentration of only one component were altered, but each component has an altered molecular ratio, implying a change in the relative concentration of at least 2 different components. From this data it was concluded that the concentrations of the components in the less viscous and original placebo formulation are not the same, as has been implied by the total dissolved solids and viscosity observations. The difference between the original and less viscous formulations was not only related to the ratio of only one component, several concentrations had been altered. This was postulated to have arisen because the aqueous solubilities of some of the constituents were exceeded at lower temperatures or high concentrations. The resulting physical separation by crystallization or removal by filtration would result in an altered constituent ratio. Adjustments were made to the formulation preparation by lower solids concentrations, and a new placebo formulation, more consistent and representative of the original formulation was obtained and used for subsequent powder production and tests.

3.4 Dry Powder Vaccine Activity Homogeneity

In the production of a dry powder measles vaccine, it was desired to demonstrate the homogeneity of the viral particles in the powder has been called to question. Ideally, vaccination is achieved by delivering particles below 5 μm in aerodynamic diameter to the deep lung and alveoli where the dry powder is quickly dissolved in the thin aqueous layer on the lung epithelium. Viral particles are exposed to the immune system through dendritic cells, macrophage scavenging or goblet cells. In order to verify this route of immunization our group sought to prove that the viral particles were in particles of our active formation at sufficient concentration, irrespective of particle size.

Fine droplet formation in an atomizing spray is an essential element of rapid drying and fine particle formation. As the high pressure emulsion of near critical CO_2 and liquid solution exits the restrictor opening, it forms a fine spray of liquid droplets with some dissolved CO_2, which quickly vaporizes, further nebulizing the liquid of interest. This is very difficult to characterize due to the fast kinetics, microscopic scale and large concentration of liquid droplets. Doubtless, in the highly concentrated spray, there are many concurrent droplet-droplet interactions that are very difficult to model. As the micro- and nano-scale droplets are exposed to the drying gas, water vapor leaving the droplet cools the remainder of the droplet by evaporation until the droplet is essentially fully dry, at which point it can come into equilibrium with the drying gas. In this case smaller particles, and the active ingredients (viral particles) in them, may lose water faster and be exposed to higher temperatures than in the larger particles which take longer to dry. Some have worried that this could lead to greater thermal degradation in the smaller particles. Smaller particles will also have larger surface areas leading to a decreased potential of full surface coverage of the active ingredient (viral particle) with sufficient excipient, which could lead to increased degradation. The following experiments demonstrate that there was no detectible difference in the viral activity per unit mass for the particle size ranges observed by ACI.

In nature, some species of bacteria have been known to aid in ice nucleation (Maki 1974) by lowering the freezing temperature of water and have been used to seed clouds. This trait was evolved by this bacteria to produce ice crystals in the cells of plant leaves, aiding access of the bacteria to the nutrients inside. While the EZ measles vaccine is not likely to have evolved this unique ability, the surface proteins and viral membrane may act to influence nucleation or hydration during the particle forming process. Additionally, the large Peclet number of a 300 nm viral particle may result in a higher surface to bulk ratio of the viral concentration. This could lead to an increased opportunity of degradation on surfaces of smaller particles where the surface area to volume is larger than that of larger particles (Burger, Cape et al. 2008)

Collection, storage and further processing, such as filling into delivery devices, may also disproportionately affect particles. Particle size differences can lead to differences in electrostatic charging, thermal resistance and sheer forces which may affect the viability of the viral particles contained within the primary particle. Smaller particles have a larger tendency to aggregate and may form larger aggregate particles that will not be differentiated from larger primary particles.

3.4.1 Activity ACI, Experimental Design

In order to determine the homogeneity of the active measles virus particles within our dry powder we designed an experiment to measure the activity of particles segregated by size range. Particles can be separated by aerodynamic diameter in a non-destructive manner by inertial separation with Andersen Cascade Impaction (ACI). ACI separates particles into size bins on plates beneath certified stages. Size separation is achieved because the flow rates increase progressively through the stages by sequentially reducing the total area of the pores inset into the stages. This causes particles with higher momentum than the calculated cut-off to impact on the plate of the stage. For our studies, a modified 5-stage ACI was used to collect the particles in different bins representing a range of size fractions.

A Westech stainless steel ACI was used for all testing. Stages 0,1,3,5 and F were used with an aluminum neck for coarse particle removal, the aerodynamic diameter cutoff (run at 28.8 L/min) for each stage is shown in Table 3.2

Stage	D_{50} Cutoff (µm) in aerodynamic diameter
0	8.7
1	6.4
3	2.7
5	0.94
F	0.44 mm glass fiber filter

Table 3.2 Diameter cutoff for each stage used in the Andersen Cascade Impactor.

As reported above, particles between 0.94 µm and 2.7 µm were collected on stage 5, and so on, with particles 8.7 µm and above impacting on stage 0 and all particles below 0.94 µm collected on the glass fiber filter at stage F. Preliminary tests used sterilized Nylon filters placed on stages 3, 5 and F, but there was significant difficulty with filter seating and airflow resistance on stage F. In the reported studies, powder was collected on ungreased stainless steel plates. Testing using vaccine placebo and greased plates showed no statistical difference from the ungreased results and the added difficulty of inaccurate gravimetric measurement, possible incomplete dissolution, and cell culture interference was avoided. Plates, stages and tools were cleaned and autoclaved, all experiments were conducted in an antiseptic environment inside a BSL II cabinet. Any testing supply that was not amenable to autoclaving was sanitized with 70 % isopropyl alcohol and ultraviolet radiation. Three PuffHaler blisters of live virus measles vaccine powder (Batch no. 68 produced at Serum Institute of India (SII)) and Plates 0,1,3,5 and a glass fiber filter were weighed before testing and placed in PuffHaler Dispersers. With a flow rate of 28.8 L/min through the ACI, three separate actuations were made into the same PuffHaler spacer and mask using a standard bulb. The spacer mask was placed over the ACI inlet and emptied before the next actuation. In all tests the same operator squeezed the bulb, for consistent actuation. Empty blisters, plates and the glass fiber filter were weighed with powder, plates were transferred to sterile Petri dishes. Each plate was washed with 3 ml of sterile PBS solution and all powder was dissolved by visual inspection, this sample was transferred to a sterile vial for viral activity assay. The final filter was not tested for activity due to difficulties with incomplete dissolution of the powder, loss of liquid to the filter and potential of loss of viral particles to the filter material. Washed and dried PuffHaler blisters were weighed to elucidate the initial fill weight of the blister.

3.4.2 Results of Activity Homogeneity Study

Viral assay testing of the material collected from each of the stages was conducted in Dr. Mark Hernandez's lab by Dr. Meg Howard. Viral content was reported in log 50% Cell Culture Infective Dose ($CCID_{50}$) per mg as determined in Vero Cells. The particle size range, determined by the cutoff diameters of each stage used, was organized into size bins and shows a distribution with a median size of 3.3 to 5.8 μm D_A , shown in Figure 3.2.

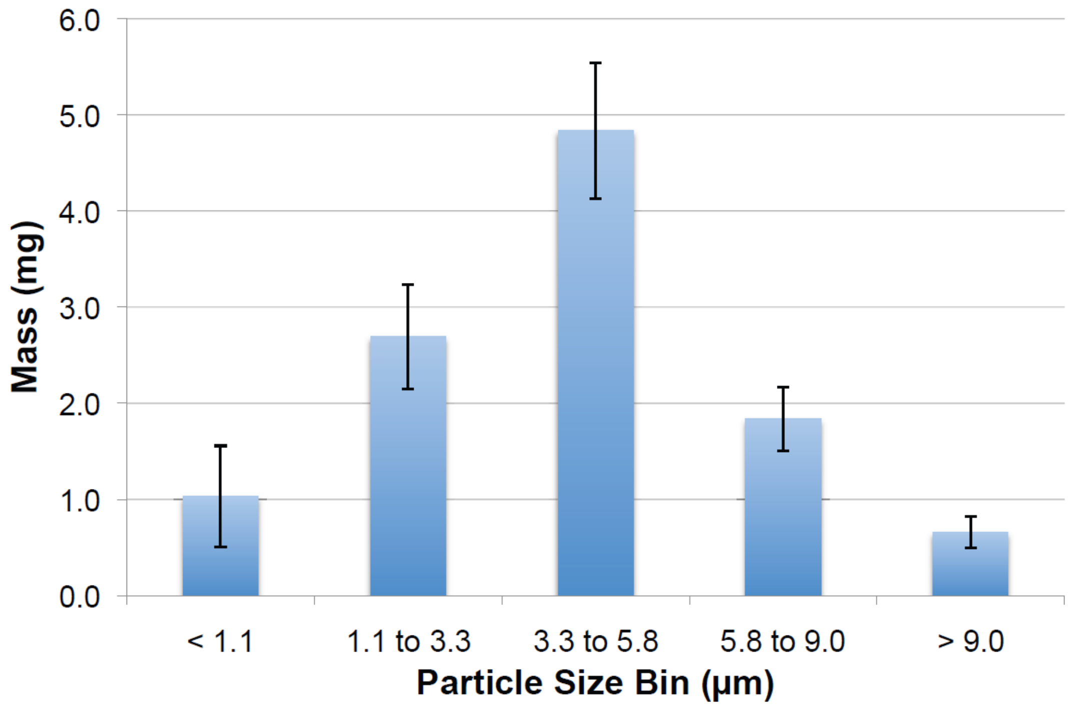

Figure 3.2 Particle size range of MVDP powder as analyzed by ACI

The viral activity was scaled with the mass of particles deposited on each stage and can be seen plotted in Figure 3.3 .

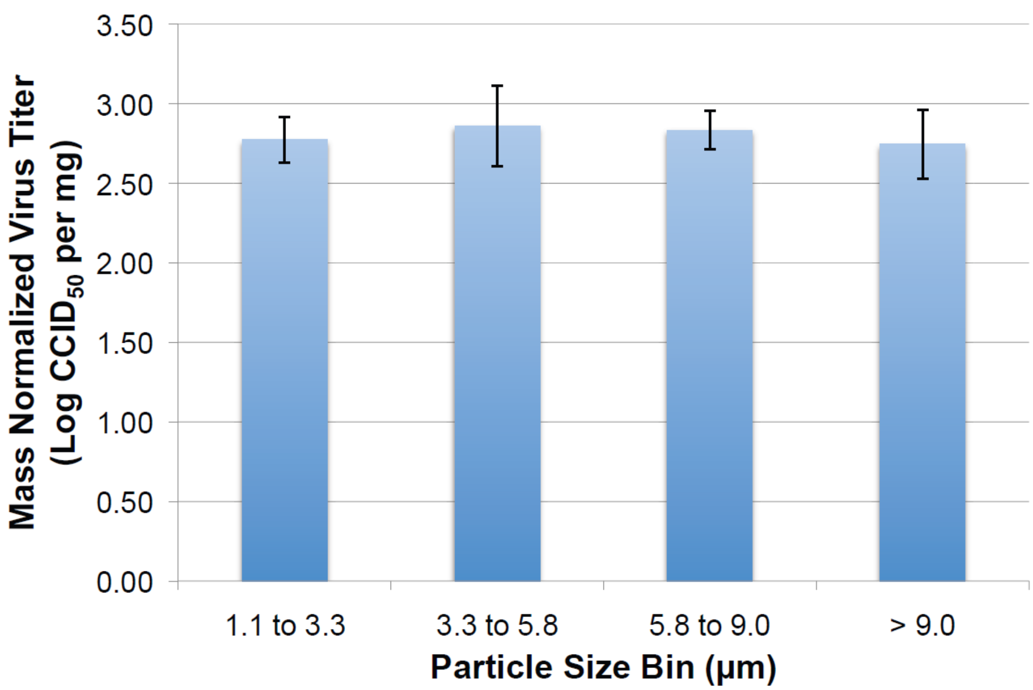

Figure 3.3 Viral activity (Log CCID50/mg powder) vs. vaccine powder particle size.

From this experiment it can be seen that the distribution of infective viral particles is homogeneous among the particles sizes observed. This implies no particle size effects due to the presence or concentration of viral particles. It also shows that there is no apparent preferential degradation of infectivity among the particle sizes observed. This shows that our dry powder measles vaccine can be effective in delivering live-attenuated measles virus throughout the pulmonary system, based on particle size-specific deposition. This particle size spread may result in more thorough deposition of the live-attenuated virus through the mucosal surfaces of the entire respiratory tract, simulating a wild-type infection and improving natural immune response.

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Chapter IV Characterization of *myo*-Inositol

4.1 Properties of Excipients

Except in very rare situations, pharmaceutical preparations are formulated with excipients to control the physical properties and increase product stability. Excipients are the inactive compounds included in pharmaceutical preparations to serve as bulking agents, or to stabilize the physical properties of the product or the activity of the pharmaceutical. The thorough characterization of potential excipients is very important in pharmaceutical chemistry and for regulatory purposes. Different properties of pharmaceutical excipients have a large influence on the utility and processing conditions that can be employed. Commonly used excipient compounds are amino acids, peptides (such as partially hydrolyzed gelatin), polymers, buffers and sugars.

For materials in which the activity of the ingredient is reliant on a particular shape and folding of a macromolecule, such as a protein or live virus, potential excipients can be defined as chaotropes or kosmotropes, based on their interactions with the water in solution and around dissolved macromolecules. Chaotropes and kosmotropes were originally described by the Hoffmeister series in 1888, a scale of common solute ions arranged in order of their experimentally determined ability to decrease or increase the solubility of protein (Crowe, Carpenter et al. 1998). Kosmotropes (e.g., carbonate anions and sulfate anions) are known to decrease protein solubility, “salting out”, and may increase protein stability. Chaotropes (e.g., thiocyanate and chromate) enhance protein solubility but can decrease protein stability (and create toxicity concerns) (Abraham, Byrne et al. 2005). The Hoffmeister effect is dependent on solute concentration and is a result of direct interaction of the solutes with the water molecules arranged at the interfacial surface of the macromolecule

(Nord, Vaag et al. 2004). A well-ordered hydration shell around a dissolved protein will lead to decreased structural denaturation during the drying process.

Some nonionic compounds, particularly those with an abundance of polar side groups, are observed to have a similar effect. This is due primarily to the strong interactions of polar bonds with the interfacial waters of hydration around the macromolecules. This effect is noted with disaccharides, maltose and trehalose, (Vehring 2008) and polyols (Vehring 2008).

Crystal formation in excipients may result in the formation of a number of different crystal polymorphs or a combination of several of these polymorphs. Crystal polymorphs, here defined as materials that differ in apparent crystal structure while retaining the same molecular identity, can have very different properties, such as solubility, instability, and biological activity, which can affect the final product. These crystal polymorphs can result from different processing parameters or storage conditions. Lactose, for instance, has four well-known crystal forms (Zhang and Cremer 2006) resulting from different crystallization techniques or natural chemical isomerizations. Each of these crystal forms shows different chemical properties, some of which may not be desirable in the final product.

In order to stabilize proteins and sensitive bioactive molecules, crystalline excipients are generally avoided, when possible. The formation of crystalline surfaces by freezing solvents or crystallization of excipients can denature proteins or lyse cell membranes at the crystal interface (Zhang and Cremer 2006). In the formulation of lyophilized and other dried products, cryoprotectants that prevent crystal formation are included to retain activity of the product during processing and storage. Some cryoprotectants and other added excipients can add additional product stability by encasing the active ingredient in a glassy matrix (Marcus 2010). Certain sugars are especially applicable to protein or live virus based formulations in that they can form sugar glasses, or amorphous matrices, under specific processing conditions.

Sugar glasses are a low viscosity state of matter, consisting of random arrangements of sugar molecules; they have a viscosity above that of the liquid phase yet below that of the solid, crystalline phase. In dried formulations with proteins or other hydrophilic molecules, it is believed that the randomly arranged hydroxyl groups of the sugar replace water molecules lost in the drying phase (Magazu, Migliardo et al. 2005). This stabilizes the hydrophilic molecule in its native form, which is important for the bioactivity or immunogenicity of that active ingredient. This phenomena is noted in nature in the high concentration of trehalose (a disacchride prone to glass formation) in the cells of certain mushrooms and the desiccated embryos of brine shrimp ("sea monkeys") which can survive periods of dehydration and revive upon the addition of water (Uedaira 2001). Lyophilized or spray dried formulations have been stabilized by the formation of glasses of sugars such as trehalose, sorbitol, sucrose and *myo*-inositol.

The stability of these sugar glasses is dependent on the temperature of storage, additional additives and humidity of the material. At temperatures 10 to 30 °C above the glass transition temperature (T_g, a characteristic temperature of an amorphous material indicated by a slight thermal transition and a viscosity change (Kirk, Dann et al. 2007), the viscosity of the glass decreases, resulting in a phase of material known as the rubbery state (Chang, Kendrick et al. 1996). The rubbery state can allow the flow of the sugar matrix, leading changes of the physical shape of the material and fusion to neighboring particles and surfaces. Potential matrix-forming excipients can be categorized as: eutectic, glass-forming or doubly-unstable.

Eutectic materials, such as NaCl, have a lower eutectic point (T_e), or temperature of crystallization, compared to their glass transition point. These materials, in the absence of sufficient interference, will tend to crystallize before they form a glass (Imamura, Ogawa et al. 2003), and are undesirable as stabilizing agents. Glass-forming materials exist as glasses when sufficiently concentrated and exhibit a glass transition significantly below their crystallization temperature. The sugars, trehalose and sorbitol, are commonly cited as good glass-forming excipients, as they can readily form glasses with Tg of 120 °C and 77 °C, respectively, and resist crystallization during brief exposures to temperatures above their T_g (Crowe, Carpenter et al. 1998). Doubly-unstable excipients can form glasses on concentration but may crystallize during processing or storage. Recrystallization can be related to the difference between material temperature and its T_g and time, meaning that extended periods of exposure to temperatures moderately above the T_g can carry the same crystallization potential as brief excursions to temperatures well above the T_g (Hengherr, Schill et al. 2011). Mannitol is a sugar alcohol classified as doubly unstable, yet it is currently investigated as a lyoprotectant or stabilizer for freeze dried and spray dried formulations (Simperler, Kornherr et al. 2006). Mannitol can only be used as a lyoprotectant in its amorphous form, so it is formulated with additional excipients; amino acids, salts and other sugars, to impede crystallization (Turchiuli, Gianfrancesco et al. 2011).

The addition of other excipients, for example; ions, amino acids and some proteins, can improve the amorphous properties of materials by inhibiting crystal formation (Gearing, Malik et al. 2010). These materials must be carefully dosed into the final formulation as they can also act as plasticizing agents, lowering the overall glass transition temperature of the formulation. One of the strongest and most ubiquitous plasticizing agents is water, which can greatly increase the T_g of a formulation in small concentrations (Hatley and Blair 1999), so hygroscopicity, or ability to take up atmospheric water, should be avoided if possible. Amorphous materials are generally more hygroscopic than their crystalline counterparts, as water molecules are more mobile within and through a disorganized glass than a well-ordered crystal (Hatley and Blair 1999). In order to avoid water uptake, dried products are typically handled in dry box environments, purged to a low humidity (<20% RH) with dry gas. Dried products should be stored in sealed impermeable containers and moisture impermeable secondary containment (such as foil overwrap) with sufficient desiccant, to remove and maintain at low levels any residual atmospheric moisture.

During our research into the formulation of a dry powder measles vaccine for inhalation, many different sugar excipients were investigated (Sadrzadeh, Miller et al. 2010). Mannitol, lactose, sucrose, sorbitol, trehalose and *myo*-inositol were all studied as potential sugar excipients. Mannitol formulations showed a tendency to crystallize after collection on the filter. Lactose, sucrose and sorbitol formulations showed undesirable hygroscopicity in the final product. Powders were formed that fit the desired specifications using both trehalose and *myo*-inositol, but ultimately, a *myo*-inositol based formulation was chosen, because the trehalose formulation showed lower relative viral stability under accelerated storage conditions, 7 days at 37 °C (Telang, Yu et al. 2003). Due to its ability to increase viral stability and maintain relatively low hygroscopicity, *myo*-inositol was thoroughly investigated as a potential excipient sugar for dry powder formulations.

4.2 *myo*-Inositol

Inositols ($C_6H_6O_{12}$, M.W. 180.16 amu) are sugar alcohol isomers of glucose that are classified as cyclitols; they consist of a 6-carbon ring with one hydroxyl group and one hydrogen bound to each carbon. Inositol has seven distinct optically inactive meso-forms (*scyllo*-, *neo*-, *epi*-, *muco*-, *l-chiro*,-*myo*- and *allo*-) and one optical isomer form (*d-chiro*-), as determined by the axial and equatorial positioning of the hydroxyl groups located around the carbon ring (Jayasundera, Adhikari et al. 2011). Of these possible isomers, *myo*-inositol is the most common in nature. *myo*-Inositol (cis-1,2,3,5-trans-4,6-Cyclohexanehexol) is arranged with 1 axial and 5 equatorial hydroxyl groups, as shown in Figure 4.1.

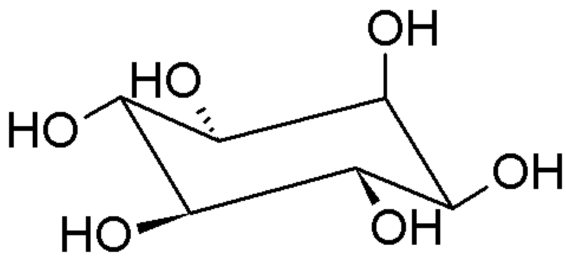

Figure 4.1 Structure of *myo*-Insoitol

4.2.1 Identity and sources of *myo*-Inositol

Ubiquitous in nature, *myo*-inositol can be consumed from many natural sources but is most concentrated in grains, nuts and fruits such as melons and citrus fruits. *myo*-Inositol is a vital nutrient in plant metabolism and factors into many chemical pathways related to plant growth (Haque and Roos 2004). Found at levels of 4-9 mg/L in normal human plasma (Naini, Byron et al. 1998), *myo*-inositol is consumed by well-nourished individuals at a natural rate of about 1 g/day and is notably a major component in human breast milk (Burger, Cape et al. 2008). This has led to its use as a an additive to infant formulas. *myo*-Inositol can also be produced through enzymatic processing of glucose phosphates in a number of human organs and tissues (Burger, Cape et al. 2008).

myo-Inositol is listed as generally recognized as safe (GRAS) by the FDA and is approved for as a food additive, nutritional supplement and for use in special dietary foods (Simperler, Kornherr et al. 2006). Tolerance of *myo*-inositol by oral ingestion has been found to be up to 18 g/day for 3 months (Loewus and Murthy 2000). This study was conducted to assess the potential of *myo*-inositol as a chemopreventative agent in smokers with bronchial dysplasia. A significant improvement in the rate of regression of preexisting dysplastic lesions was noted in patients receiving oral *myo*-inositol vs. the placebo group (91% to 48%). An additional interesting and potentially valuable side effect observed in the patients receiving *myo*-inositol at the 18 g/day level was a reduction in the systolic and diastolic blood pressure by an average of 10 mm Hg (Kusmierz, Degeorge et al. 1989).

4.2.2 Biological Activity of *myo*-Inositol

myo-Inositol has been noted to play a significant role in the female reproductive system, specifically in that it has been observed to affect fertility, oogenesis, and has been shown to have a therapeutic effect when given orally to women who suffer from polycystic ovary syndrome (PCOS) (Hallman, Bry et al. 1992). While there are yet to be universally agreed upon diagnostic symptoms of PCOS, it is thought to be the most common endocrinopathy observed in women (Pereira, Baker et al. 1990). Symptoms may include hyperandrogenism, polycystic-appearing ovaries, obesity, irregular ovulation and irregularities in estrogen and insulin regulation. Studies have found that regular oral doses of inositol can result in decreased blood-pressure, lowered blood triglycerides, improved insulin regulation, reduction of hirsuitism, and improved ovulation regularity in women diagnosed with PCOS (FDA 2011).

myo-Inositol is a relatively strong and mobile osmolyte, a compound which regulates osmosis and water transport, which are known stabilizers of membranes and proteins (Lam, McWilliams et al. 2006). The potential for a material to stabilize a protein can be approximated by its dynamic hydration number (DHN), a physical quantity related to the enthalpy of hydration of a solute. When compared with similar polyols, the presence of *myo*-inositol, in solution with several model proteins, has been found to result in a consistently larger increase in the temperature of protein degradation. This may be due to the DHN value of 18.6 for *myo*-inositol as compared to the DHN values of 16.9, 16.2 and 9.9 for sorbitol, mannitol and glycerol respectively (Lam, McWilliams et al. 2006).

4.2.3 Sources of *myo*-Inositol

myo-Inositol is commercially derived from grains and by-products, such as rice hulls, in the form of phytic acid and phytin (inositol hexaphosphoric acid and its calcium-magnesium salt), which is found in rice bran at levels as high as 57 mg/g (Lam, McWilliams et al. 2006). Phytic acid and phytin are commercially separated from the rice bran and are then hydrolyzed, usually by phytase enzymes which remove the six phosphate groups bound to the carbons of the inositol ring (Papaleo, Unfer et al. 2009).

The primary supplier of *myo*-inositol for this research was Tsuno Rice Fine Chemicals Co. Ltd., located in Wakayama, Japan. The *myo*-inositol provided by Tsuno was a coarse white crystalline powder, with a larger primary particle size than the material commercially available from Sigma-Aldrich. The Tsuno stock material was analyzed by powder XRD (Figure 4.2) and NMR, and found to be pure *myo*-inositol by these methods by comparison with literature values (Table 4.1).

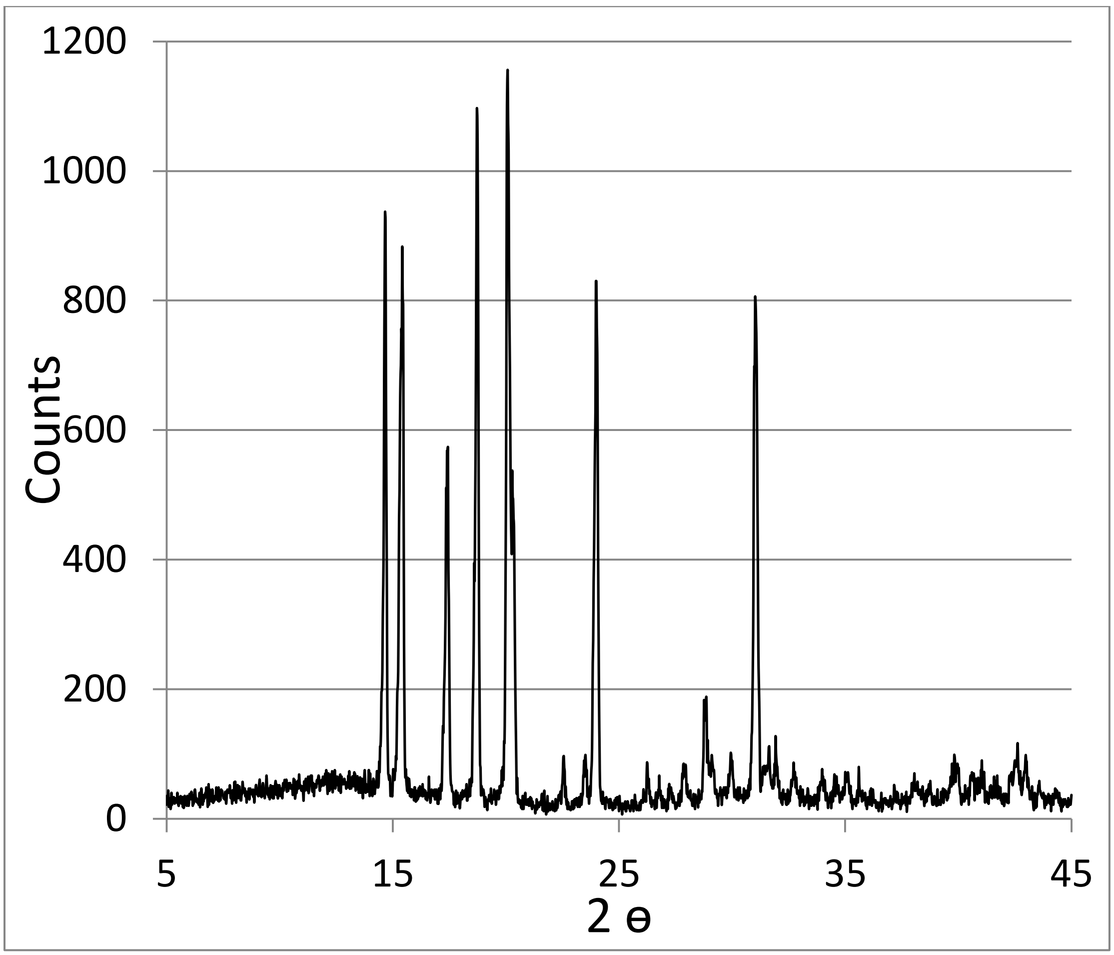

Figure 4.1 pXRD of Tsuno Stock *myo*-Inositol

Additional comparison studies were conducted on other sources of *myo*-inositol: Sigma-Aldrich, a source from China and a commercially available health food variety, all sources were found to be chemically identical by pXRD. The bulk *myo*-inositol used in our testing was purchased in a 25 kg lot and was stored throughout the 5 years of this study without detectable change in a large, plastic-lined, sealed fiber drum with exposure to the lab atmosphere only during the transfer of 1 kg amounts to a satellite container for lab use.

4.3 *myo*-Inositol Toxicity Study

In the formulation of a dry powder measles vaccine for delivery by inhalation the primary stabilizing excipient used was *myo*-inositol as 50% of the dry weight of the final formulation (Burger 2008). Powders, processed by CAN-BD, showed superior activity retention of the *myo*-inositol based vaccine powder as compared other formulations using different sugar excipients. The *myo*-inositol based formulations also show lower moisture content under the same processing parameters. While *myo*-inositol is a promising excipient, biologically ubiquitous and tolerated at high doses by oral delivery, thorough testing of the toxicology by inhalation had not been conducted at the outset of this study. As our dry powder measles vaccine was intended for human and test animal inhalation, toxicology studies of the inhaled excipient were required to progress to more advanced in vivo trials of our product. In November, 2011, approval was given by the Drugs Controller General of India to the Serum Institute of India, Ltd., our collaborators in the Grand Challenges in Global Health Initiative, to begin the Phase I clinical trials in human volunteers in Pune, India of dry powder measles vaccine aerosols stabilized by *myo*-inositol. This has followed review of extensive testing of *myo*-inositol-containing samples (formulated at the University of Colorado) in rats and macaques by collaborators in our team. An inhalable *myo*-inositol powder was designed to elucidate the toxicity, if any, of inhaled *myo*-inositol.

In powder preparation it became immediately apparent that *myo*-inositol could be qualified as a doubly-unstable compound. Powders of pure *myo*-inositol produced by the CAN-BD process were invariably crystalline and proved to be poor models for inhalable powders, because, from the dry powder inhalers employed, the % mass delivered below 5.8 μm D_A was uniformly low. In order to produce a more efficacious and finer inhalable powder, various changes were made in the processing parameters: outlet temperature, drying gas flow rate, feed flow rate and feed concentration (Table 4.1)

Process Parameter	feed concentration	feed concentration 2	feed concentration 3
	5% (m/v)	10% (m/v)	1% (m/v)
Liquid Flow Rate	%< 5.8 μm	%< 5.8 μm	%< 5.8 μm
0.3 mL/min	6.46	5.73	3.92
0.5 mL/min	7.07	8.38	n/a
Outlet Temp			
50 °C	9.80	7.59	n/a
70 °C	5.21	6.79	3.92
Drying Gas			
30 L/min	5.66	8.15	n/a
40 L/min	5.21	5.10	3.92

Table 4.1 Average % particles by mass < 5.8 μm DA for pure *myo*-inositol under different CAN-BD processing parameters

In these pilot experiments with pure *myo*-inositol, no one parameter or combination of several was found that produced a powder with sufficiently small particle size (at least 35% particles by mass< 5.8 μm D_A) to prepare a powder representative of our measles formulation, which is stabilized with 50 g/L of *myo*-inositol in the aqueous vaccine feed stream for CAN-BD drying and micronization..

An additional excipient was required to produce a suitable powder, but in order to remain representative of the initial formulation we chose potential additive excipients from those already present in the dry powder measles vaccine formulation, reported in Table 4.2. Additionally, this formulation was known to form an amorphous matrix, when dried by CAN-BD .

Component	Liquid concentration	mass % total solids
	g/L	%
myo-inositol	50	46
Gelatin (hydrolyzed)	25	23
L-arginine-HCl	16	15
L-histidine	2	2
L-alanine	1	1
Lactalbumin hydrolysate	4	3
Tricine	3	3

Table 4.2 Concentration of formulation components in dry powder measles vaccine (MVDP3)

Powder made using similar amounts of L-arginine and L-histidine only resulted in the immediate crystallization of the powder on the surface of the filter, blocking the filter pores and stopping the process run. The same results were noted following the addition of mannitol, which is another sugar alcohol used in inhalable powders (Carmina and Lobo 1999). The addition of gelatin and lactalbumin was found to produce good powder with a FPF of about 35% <5.8 μm and 13%<3.4 μm. Lactalbumin at a 1:20 ratio to *myo*-inositol was chosen as a promising additive due to the variability and hygroscopicity of gelatin.

Lactalbumin, consisting of peptides considerably larger than *myo*-inositol likely resulted in better performing particles as the higher Peclet number of the peptides would reduce their mobility, leading to a surface coating of lactalbumin on the microparticles being formed. This would prevent the fusion of the crystalline surface of *myo*-inositol particles to form aggregates (Costantino, Minozzi et al. 2009) as was seen in SEM images of pure *myo*-inositol, Figure 4.3. Long runs for bulk production of the *myo*-inositol/lactalbumin were attempted, but generally resulted in the collection of poorly dispersible flakes from the dual filter back pulse system. A higher flow rate of nitrogen drying gas was used and the chamber temperatures were increased from 50 to70 °C, but the problem persisted and the resulting flakes were shown to be poorly dispersed. Long production runs were conducted using a different filter system but still resulted in lower than acceptable FPF (28% <5.8 μm, 7% <3.4μm). SEMs of these powders have all shown small individual particles aggregated to form larger clusters, Figure 4.4. The problem with these powders appears not to be the individual particle size, but particle/particle attractive forces that hold the powder in aggregates. This was probably due to adhesion of the lactalbumin surface coating and was seen to be exacerbated at higher humidities.

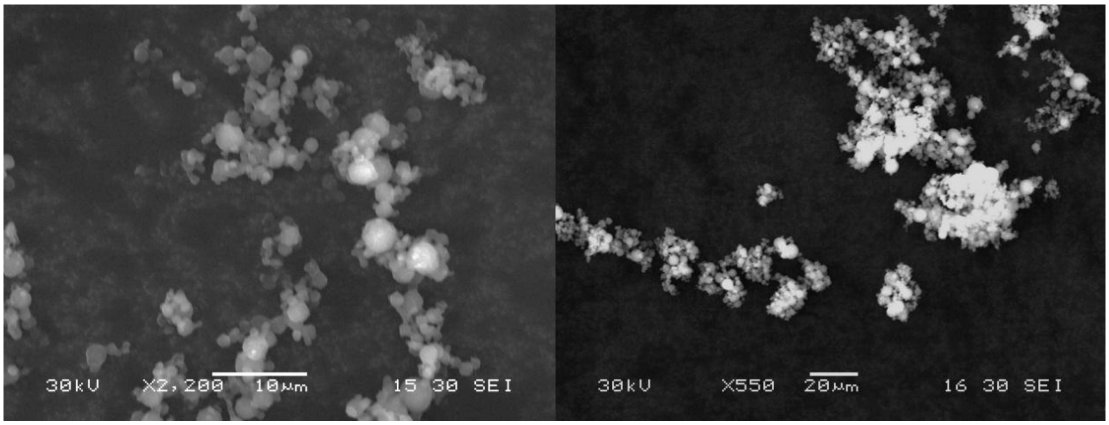

Figure 4.3 SEM Image of CAN-BD Processed Pure *myo*-Inositol, fused particles and the resulting particle aggregates

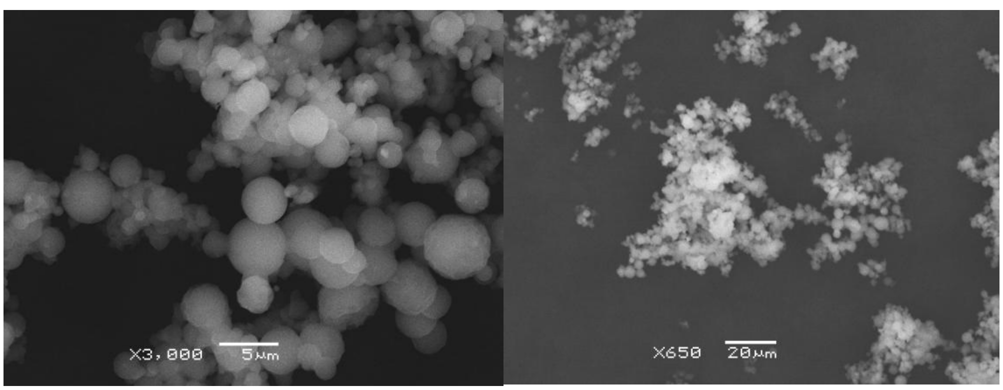

Figure 4.4 SEM Images of CAN-BD Processed *myo*-Inositol/Lactalbumin (20:1) and powder clusters

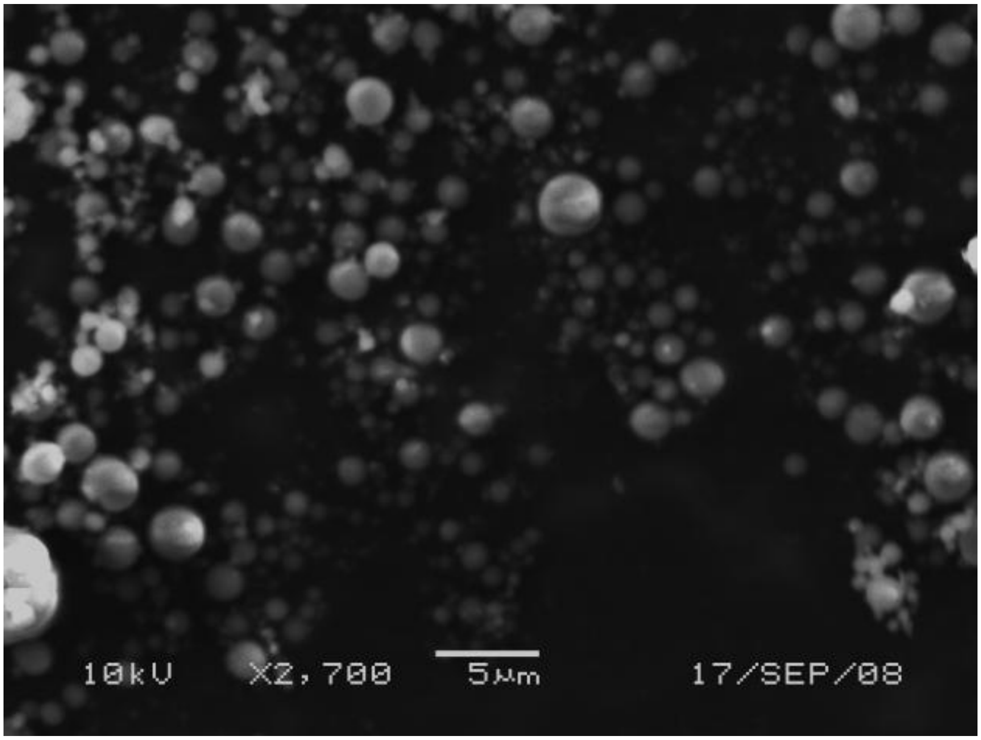

Figure 4.5 SEM Image of CAN-BD Processed *myo*-Inositol/l-luecine (98.5:1.5)

myo-Inositol powder with respirable fractions sufficiently abundant to serve as a representative powder were eventually produced through the addition of l-leucine at a 1.5%/98.5% ratio with *myo*-inositol, as seen in Figure 4.5. Other ratios (i.e. 10%/90%, 5%/95% and 1%/99%) were tested but the ratio of 1.5%/98.5% was chosen as it produced a well-behaved amorphous powder without adding an excess of the additional excipient (l-leucine). l-Leucine is a well-known excipient that is frequently used in particle engineering to reduce particle aggregation (Auton, Rosgen et al. 2011). It is thought that the insolubility of l-leucine results in the formation of an l-leucine shell around the particle where the isobutyl side chain aids in maintaining particle separation. This is supported by the change in the observed surface roughness in particles with increased concentrations of l-leucine. *myo*-Inositol/l-leucine particles shown in Figure 4.6 were prepared at a ratio of 9:1 and demonstrate much more irregular surfaces and shapes when compared to *myo*-inositol/l-leucine powders at a ratio of 98.5:1.5 in Figure 4.5.

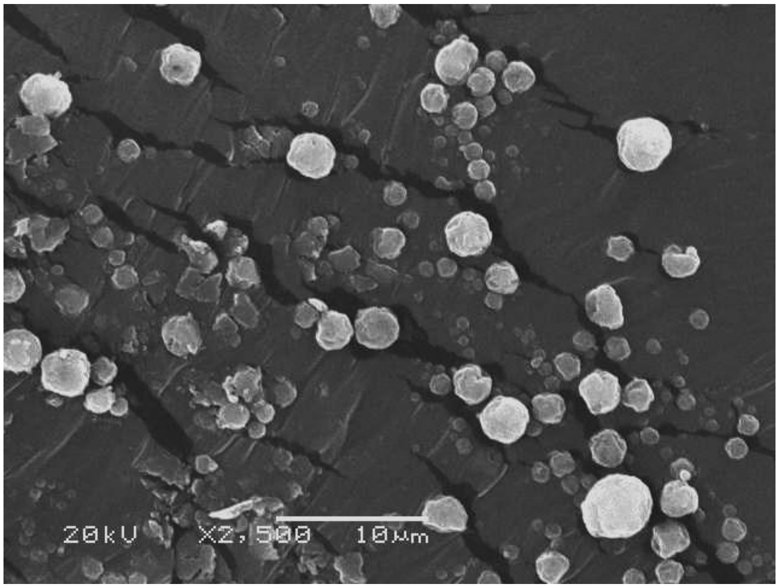

Figure 4.5 SEM Image of CAN-BD Processed *myo*-Inositol/l-leucine (9:1)

A 10 to 15 g batch of inhalable powder, consisting of *myo*-inositol and l-leucine at a 98.5/1.5 weight ratio was produced under sterile conditions for inhalation toxicity testing in Sprague-Dawley rats. This powder was tested against a placebo powder prepared from l-leucine and lactose, a sugar already FDA-approved excipient for inhalation drugs. Aerosolized powder was presented to a group of test rats at a dose level up to 30 mg/day for 3 days by an external contract toxicology testing laboratory. Powders were aerosolized into a powder reservoir and inhaled at-liberty by the test animals. Emitted dose tests at the typical inspiration rate for a rat (0.3 L/min) estimated the dose delivered to rats at a maximum of 10 mg/Kg. No adverse effects were noted for any of the dosage groups, indicating that the no-observed effect level for rats is an exposure of 30 mg/day, while a larger dose may be tolerated. Animal studies were conducted at Bridge Laboratories (now named Avanza in Gaithersburg, MD). These tests provided evidence that *myo*-inositol can be well tolerated as an inhalable excipient and have led to further testing of our *myo*-inositol-based dry powder measles vaccine.

4.4 *myo*-Inositol Crystal Polymorph

Testing of the *myo*-inositol formulations of the measles vaccine showed it to be amorphous with a glass transition temperature of about 61 ºC, but *myo*-inositol processed by CAN-BD with fewer additional solutes, specifically without gelatin, can form crystalline *myo*-inositol. It was found that different processing variables could lead to the formation of two different crystal polymorphs of pure *myo*-inositol: the previously studied common form of *myo*-inositol and a metastable polymorph, *myo*-inositol β.

The metastable polymorph, as well as the more stable polymorph, was observed at CAN-BD drying gas temperatures of 50 °C, while at 60 °C only the more stable polymorph was observed to be present. When the powder containing a mixture of the metastable and stable polymorphs was heated to 120 °C, the annealed powder no longer shows the presence of the metastable polymorph. This loss of the metastable polymorph is also noted when the mixed powder was stored at room temperature for an extended period of time (several months), without any external heating applied after CAN-BD drying at the lower temperature was completed.

Measurement of powder X-ray Diffraction was conducted on the various samples according to the methods described in the Methods chapter; scans were run from 2 Theta of 5° to 45°. Differential Scanning Calorimetry was conducted as described in the Methods chapter, from with analysis from –20 ºC to 250 ºC at scan rates of 10 ºC/min and 100 ºC/min. *myo*-Inositol powder processed by CAN-BD with drying gas below 60 ºC was analyzed by powder X ray Diffraction (pXRD) and found to have a significantly different diffraction pattern than the diffraction pattern of the unprocessed material, as shown in Figure 7. The most significant reported peaks from the International Center for Diffraction Data database (Uedaira 2001) are shown in Table 4.3.

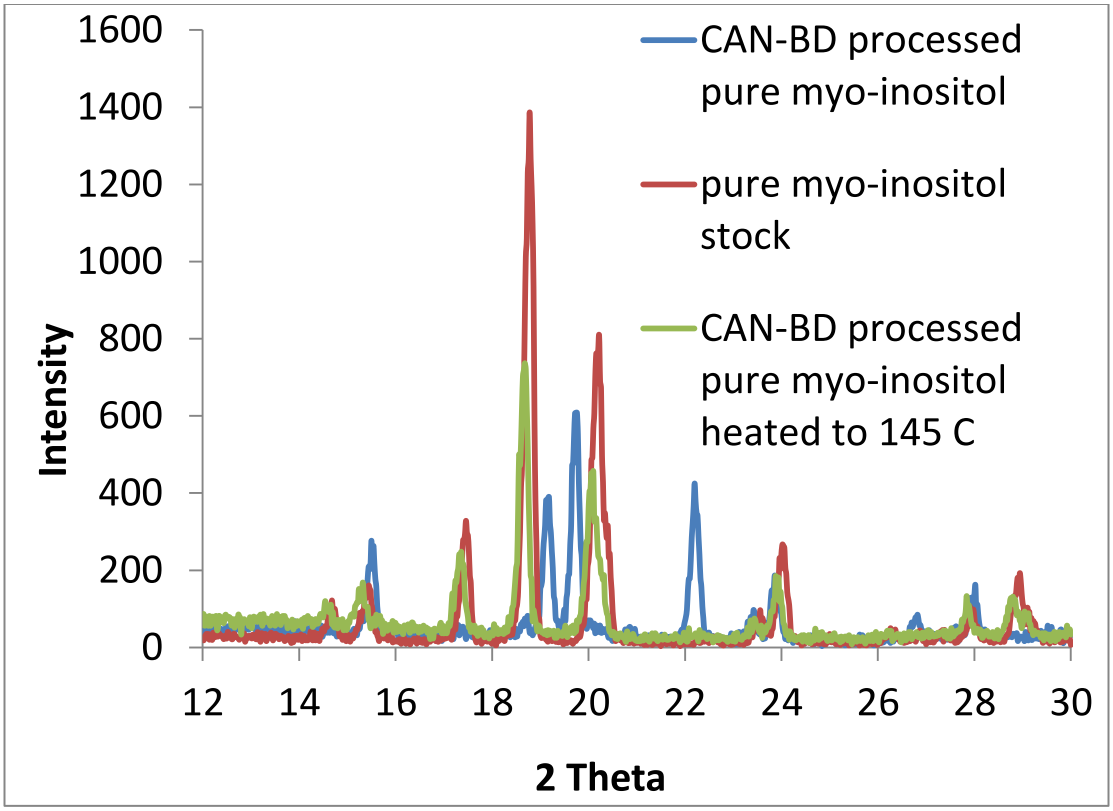

Figure 4.7 pXRD Diffractograms for different crystal forms of *myo*-inositol.

2 Theta	relative intensity	2 Theta	relative intensity
14.7	20	27.9	16
15.4	30	28.9	45
17.5	70	31.1	45
18.8	100	31.5	20
20.2	90	31.6	20
23.5	20	34.6	14
24.0	50	35.1	18

Table 4.3 Peak values expected for pXRD analysis of *myo*-inositol

It can be seen in Figure 4.7 that, in comparison to the stock material, the metastable crystal form exhibits three peaks at 2 Theta of 19.1, 19.7 and 22.2 and the major peaks at 2 Theta of 14.7, 18.8 and 20.2 are absent. Heating the powder to 145 °C causes the metastable crystalline form to revert to the more stable native crystal form. These diffraction data were consistent for all batches of CAN-BD processed pure *myo*-inositol powder dried below 60 °C when measured directly after processing, but over time the metastable crystalline form slowly reverts to the native crystal form, resulting initially in a powder with the X-ray diffraction pattern of both crystal forms, Figure 7, and ultimately in only the more stable form.

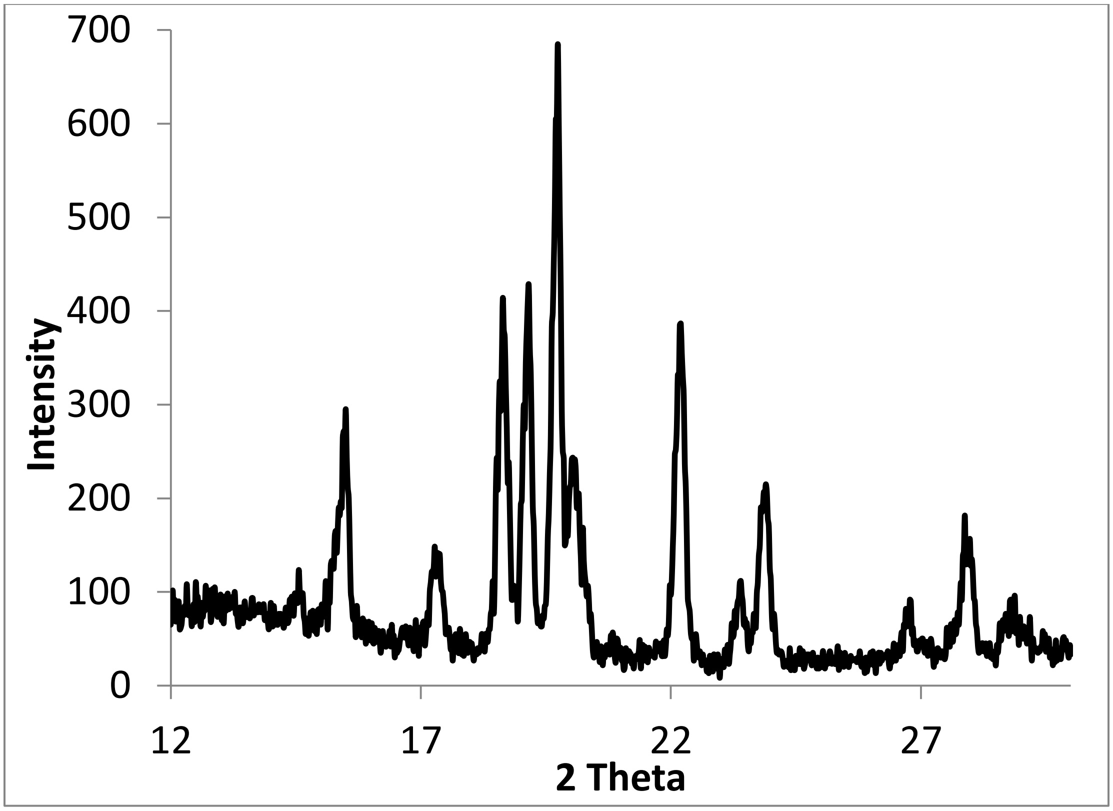

Figure 4.8 pXRD Diffractogram of aged *myo*-inositol polymorph powder showing both characteristic patterns

Study of the thermal properties of *myo*-inositol β powder by differential scanning calorimetry (DSC), as seen in Figure 4.9, shows an exothermic transition with an onset at 110 °C and a peak at 125 °C before the melting point at 225 °C , which is the literature value for *myo*-inositol (Uedaira 2001). Powder heated above115 °C no longer exhibits any endothermic transition before melting at 225 °C.

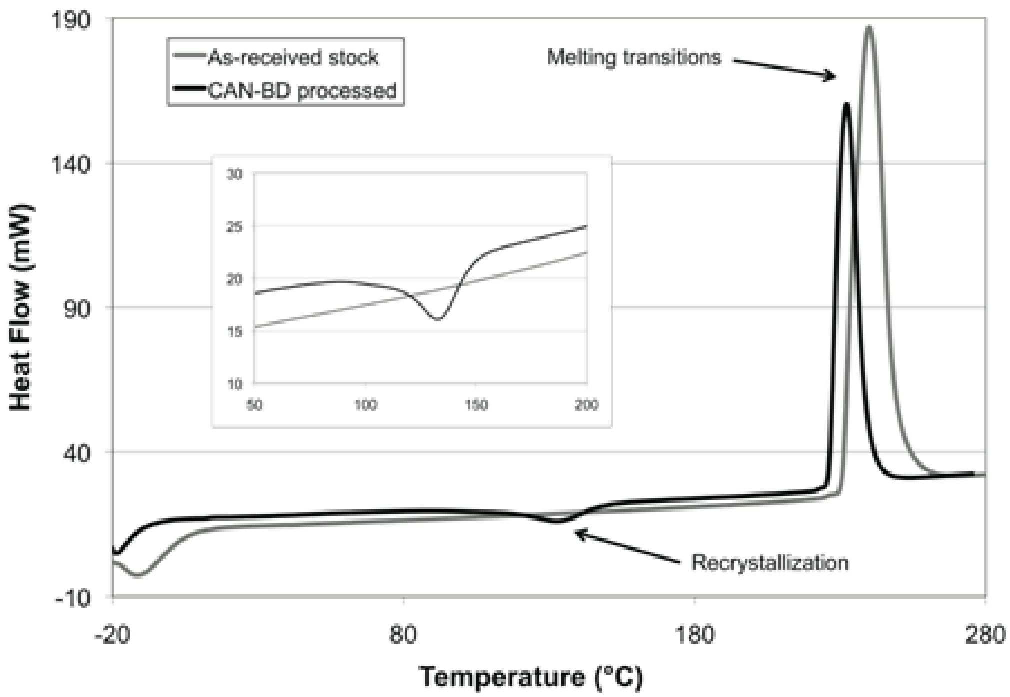

Figure 4.9 DSC thermal analysis of *myo*-inositol β crystalline powder indicating recrystallization exotherm

The moisture content of the *myo*-inositol β powder has been determined to be nearly the same as the moisture content of *myo*-inositol powder in the native form (about 0.1%). A very small mass loss was noted after heating the *myo*-inositol β powder to 145 ^{o}C. Analysis of SEM images of both show no obvious differences.

Samples of *myo*-inositol β powder have been analyzed by a differential vapor sorption instrument (DVS) by Surface Measurement Systems Ltd UK. Samples were held at 40 ^{o}C and exposed to air flow at controlled steps of relative humidity. The sample was allowed to equilibrate at each step and the percent change in mass from the initial fill weight was recorded. Steps in RH were increased from 0 % to 95 % in 5 % increments and then decreased from 95% to 0% in 5% increments. A small loss of about 0.025% was noted at about 19 % relative humidity for each run. A graphic representation of this analysis can be seen in Figure 4.10. Overall, the powder gained 0.2% mass or less over the course of RH increase, with the largest gain being at 95 % RH. The mass gained during humidification of the powder was lost upon the decrease of RH to 0% RH.

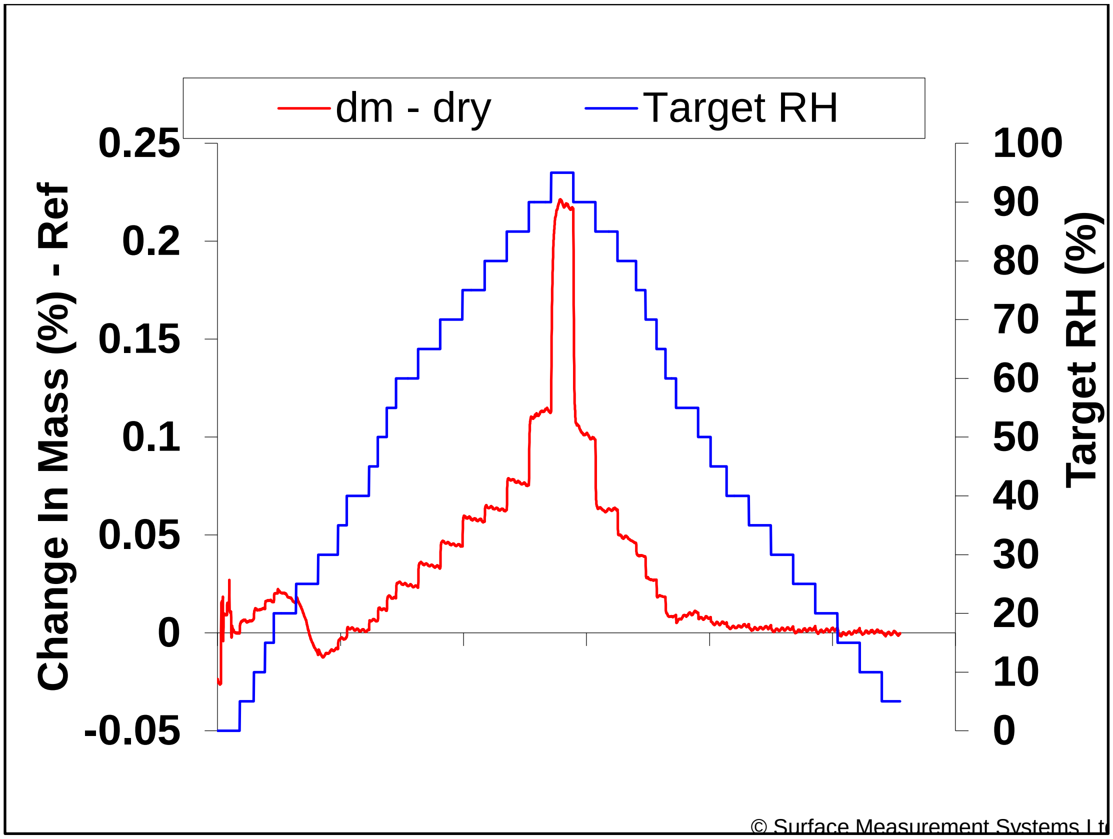

Figure 4.10 Differential Vapor Sorption (DVS) study of the meta stable crystalline *myo*-inositol

Diffraction data taken of earlier-prepared (older) *myo*-inositol β powders stored and room temperature, and of freshly prepared *myo*-inositol β powders heated to 145 °C (Figure 6) show a reversion to the common crystal form. Samples of *myo*-inositol β powders stored at 2-8 °C retain the unique pXRD diffraction pattern and the exothermic event at 110 °C for up to 5 months.

Because *myo*-inositol is being tested as a new sugar stabilizing excipient, its various crystal polymorphs should be characterized and categorized. A *myo*-inositol dihydrate has been previously reported (Garcia-Estepa, Guerra-Hernandez et al. 1999) but our *myo*-inositol β powders are not the dihydrate from. The moisture content of the *myo*-inositol β powders is very low (0.1 % water) and statistically equivalent to the moisture content of the native *myo*-inositol. DSC studies of the *myo*-inositol β powders show no endothermic dehydration event before the material melts at 225 °C, where the dihydrate is expected to melt at 196 °C (Greiner, Alminger et al. 2002). Additionally, the powder XRD pattern of the *myo*-inositol β powder does not match that of the published calculated *myo*-inositol dihydrate pattern (Sadrzadeh, Miller et al. 2010). Therefore, it can be reasonably concluded that there are no detectable amounts of the dihydrate present in the well-dried *myo*-inositol stabilized samples examined in the present study.

Samples of *myo*-inositol β powders that were heated past the exothermic transition at 120 °C without melting revert to the native form of *myo*-inositol, based on X- ray diffraction. Heating these samples shows a very low loss in mass that is not statistically different than that of native *myo*-inositol powders, indicating that the difference between these two is solely related to the change in crystal structure.

DVS results for the powder showed a slight decrease in mass (about 0.02%) after increasing air RH to 20%. This is likely due to plasticization of the amorphous content of the powder and subsequent crystallization of that portion, resulting in the loss of water adsorbed to the previously amorphous content. Most substances defined as crystalline still have some amorphous content, which may go unnoticed by some methods of crystalline content analysis (pXRD) (Adhikari, Howes et al. 2009). Due to its relatively disorganized state, an amorphous sugar is usually more susceptible to moisture uptake than a purely crystalline form of the same sugar (Vehring 2008). The addition of water may act to decrease the glass transition of the material and can lead to crystallization of the amorphous material (Rudman 1962), resulting in the loss of water adsorbed to the glassy material. A large % mass gain would be expected for an amorphous form of *myo*-inositol during exposure to elevated RH and so the DVS results imply a low amorphous content of the *myo*-inositol β powder.

The pXRD diffraction pattern of *myo*-inositol β is not likely due to sample preparation artifacts. Preferred orientation is very unlikely as the particles are in the micron and sub-micron range and are randomly arranged. While there are no reported powder XRD diffraction patterns for *myo*-inositol polymorphs, there are some single crystal diffraction patterns of *myo*-inositol polymorphs (Rudman 1962). Comparison between our powder X-ray diffractograms and the single crystal diffraction patterns is very difficult and single-crystal XRD was not pursued for our material due to the difficulty of separating out one particle and the possibility of microcrystalline structure.

The metastable crystal polymorph noted for CAN-BD processed *myo*-inositol dried at temperatures less than 60 °C may be evidence of a *myo*-inositol in its energetically unfavorable chair formation. *myo*-Inositol is known to have two possible chair conformations (A and B) seen in Figure 4.11. In crystalline form, calculations based on the theoretical coupling constants of each conformation show that chair conformation A is preferred by 30 kJ/mol over chair B resulting in a theoretical mole ratio of 99:1 (A:B) (Simperler, Watt et al. 2006).

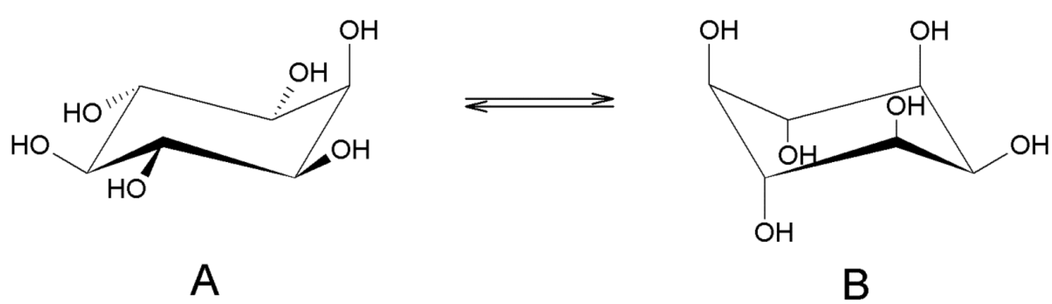

Figure 4.11Structure of the favored (A) and unfavored (B) chair conformations of *myo*-inositol.

The CAN-BD spray drying process, with lower drying temperatures, may lead to the formation of a near pure crystalline powder of the thermodynamically unfavored (but kinetically accessible) chair configuration, but further computational analysis will be required to prove this. Models of infinite hydrogen bond chains of *myo*-inositol show that *myo*-

inositol crystals follow an irregular double chain parallel pattern where molecules sharing two OH bonds with a neighbor molecule alternate with molecules sharing only one OH bond (Bonnet, Jones et al. 2006). The irregular crystal pattern, in conjunction with an additional possible chair conformer may explain the added stabilization properties of *myo*-inositol, where a crystalline matrix can be sufficiently irregular to provide the same benefits as an amorphous material. Still, as exemplified by the metastable character of CAN-BD processed *myo*-inositol, care must be taken to prevent the crystalline rearrangement of *myo*-inositol in a formulation.

4.5 Differentiation from *scyllo*-Inositol

Of the eight different isomers in the inositol family, the second most common naturally found stereoisomer of inositol is *scyllo*-inositol. Originally derived from the kidney of dogfish (genus Scyllium), it is also found naturally in acorns (Bonnet, Jones et al. 2006). *scyllo* -Inositol has been identified in healthy brain tissues at concentrations of 0.3 mM for young subjects and 0.43 mM for older subjects (Terakita, Matsunaga et al. 2009). Studies have shown transgenic mice with Alzheimer's disease treated with oral *scyllo* -inositol have shown a decrease in neurotoxic Aβ oligomers in the brain, leading to the inhibition of typical behavior defects (Ward and Schultz 1995). In 2009, Elan Corporation, plc and Transition Therapeutics, Inc. were conducting clinical trials of orally delivered *scyllo* -inositol in human Alzheimer's patients, and, as of Dec 2009, ended the higher dose patient groups (1000 mg and 2000 mg dosed twice daily) due to possible adverse effects (Hancock and Dalton 1999).

Due to speculation, subsequently proven to be groundless, that *myo* -inositol previously used for powder production might have contained small amounts of *scyllo* -inositol, high field, high resolution NMR analysis was conducted to confirm the purity of stock *myo* -inositol, in respect to the stereoisomer *scyllo* -inositol. *scyllo* -Inositol has a reported melting point of

325 ºC (Hancock and Dalton 1999) which is significantly higher than the melting point observed for the stock *myo* -inositol, 225 ºC. *scyllo* -Inositol is a carbon symmetric molecule with only one singlet by ^{1}H NMR at 3.34 ppm (Khan, Qureshi et al. 2007), structure shown in Figure 4.12.

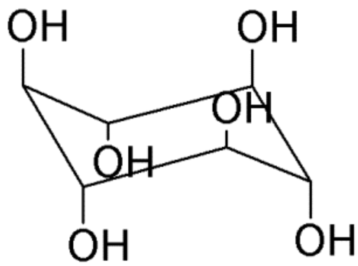

Figure 4.12 Structure of *scyllo*-inositol

myo -Inositol by contrast has expected peaks at 3.259 ppm, 3.282 ppm, 3.305 ppm, 3.523 ppm, 3.53 ppm, 3.548 ppm, and 3.555 ppm (SalazarPereda, MartinezMartinez et al. 1997). The ^{1}H NMR spectra of stock *myo* -inositol in D_2O shows the expected peaks for *myo*-inositol, with no peaks at 3.34 ppm, Figure 4.13. This shows that no detectable *scyllo* -inositol is present in our stock *myo* -inositol as determined by high field ^{1}H NMR. The conversion process for *myo*-inositol into *scyllo*-inositol is quite involved (Simperler, Watt et al. 2006) and very unlikely to occur during processing or storage of our formulations; furthermore, there is no indication of any such conversion.

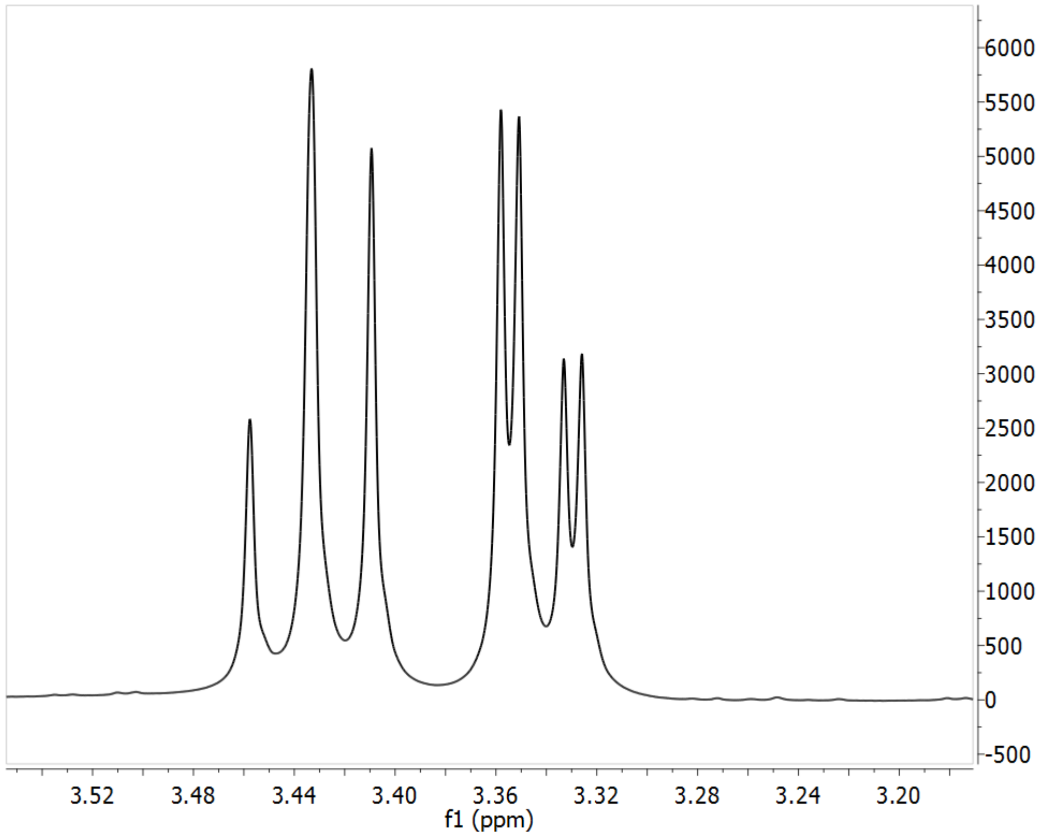

Figure 4.13 1H NMR spectra of stock *myo*-inositol in D_2O (3.20 ppm to 3.52 ppm)

4.6 *myo*-Inositol Conclusions

While it appears to be a doubly unstable sugar, only resulting in crystalline material when processed on its own, *myo*-inositol may prove to become a beneficial new excipient. Difficulties in particle formation were overcome through the addition of complementary excipients and a toxicology study on the prepared powder showed inhaled *myo*-inositol to be well tolerated in rats at exposures of up to 30 mg/Kg per day for 3 days. A metastable crystalline form of *myo*-inositol was produced in a near pure form, as assayed by pXRD, by CAN-BD processing. This polymorph may prove to consist of an extensive crystal of the thermally unstable chair conformer of *myo*-inositol. Additionally, characterization techniques, using pXRD, NMR and DSC, were developed to determine the identity and purity of *myo*-inositol in formulations and both possible crystal forms.

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Chapter V Development of a Dry Powder Human Papillomavirus Recombinant Protein Vaccine

5.1 Human Papillomavirus, Current Vaccines

Human Papillomavirus (HPV) is currently the most prevalent sexually transmitted infection in the US. Approximately 20 million Americans are currently infected and 5.5 million more become infected each year (Simperler, Watt et al. 2006). There is evidence that at least 75% of sexually active individuals will be infected with at least one serotype of HPV over their lifetimes (Witczak 2008). Over 100 serotypes of HPV have been identified, the majority of which carry a low risk of severe symptoms. Infections may be asymptomatic or may cause warts and lesions and can lead to cervical, penile, anal or throat/neck cancer. Some studies have found HPV virus in up to 61% of neck and head cancer samples (Kaiser, Schuff et al. 2005). HPV is the primary cause of cervical cancer, the second leading cause of cancer deaths in all women. One recent study shows that cervical cancer in rural India may even be twice as common as breast cancer (McLaurin, Kierstead et al. 2006). In the US, 10,000 women are diagnosed with, and 4,000 women die of, cervical cancer every year (Salloway, Sperling et al. 2011). Worldwide, there are half a million cases of cervical cancer, leading to a quarter million deaths every year (Day, van de Streek et al. 2006).

In recent years, effective but expensive vaccines for HPV such as Gardasil and Cervarix have gained approval in the US and abroad. Gardasil consists of recombinant, self-assembled, virus-like particles (VLPs) made up of HPV L1 capsid proteins of the HPV types 16, 18 (which cause 70% of cervical cancer) and 11 and 6 (which cause 90% of genital warts). Cervarix contains the VLP antigens to only HPV16 and 18. Gardasil is recommended for girls age 11 to 26, although it may be given to girls as young as 9. The cutoff of approval at 26 years of age was instituted due to the near ubiquity of HPV in sexually active women. Any sexually-active woman over the age of 26 is likely to have been exposed to one of the target varieties of HPV and the immunogenic benefit to the recipient is considered to be insufficient to justify vaccination of any woman above that age. Gardasil is a prophylactic vaccine and is not considered therapeutic. Gardasil has recently been approved and shown to be effective at protecting males from HPV infection (Kaiser, Schuff et al. 2005). Vaccination of males may or may not provide direct and significant benefit to the male recipient, but should reduce the spread of HPV (as well as protecting the man from the rarer incidences of penile, neck, head and anal cancer as well as genital warts). For these reasons, on Oct 25th 2011 the Advisory Committee on Immunization Practices released a recommendation that all males, in addition to females, aged 11 to 12, be vaccinated for HPV (Kaiser, Schuff et al. 2005).

The currently approved vaccines are formulated as liquid suspensions, adjuvated with amorphous aluminum hydrophosphate sulfate, in the case of Gardasil, and aluminum hydroxide and a proprietary lipid adjuvant, in the case of Cervarix (SDBSWeb). These vaccines are packaged as 0.5 ml liquid vaccine in single dose vials and need to be stored between 2 and 8 °C; elevated temperatures may lead to bacterial growth or protein degradation and freezing may lead to vaccine deactivation or separation and precipitation of the adjuvants. The vaccines are given in a 3-dose series over 6 months in order to give maximum protection (Husson, Odier et al. 1998). Due to reports on the efficacy and necessity of this vaccine, many states are considering instituting mandatory HPV vaccination for school girls as young as 11. Some states urge vaccination but offer an opt-out. Due to the frequency and ease of infection, the effectiveness of these vaccines are at a maximum when they are delivered to women before they become sexually active.

The VLPs used in the current vaccines are made up of 72 L1-capsid protein pentamers which self-assemble to form virus-like spheres, without the L2 capsid protein and viral genome, thus providing no risk of infection (Moscicki 2005). In order to produce these self-assembled VLPs, the L1 proteins are expressed in and purified from insect cells (Cervarix) and yeast (Gardasil) and subsequently extracted and purified. Research by Warzecha et al in 2003 shows that HPV L1 proteins can be expressed in transgenic potatoes and these proteins have been shown to self-assemble into VLPs (Warzecha, 2003). What is particularly interesting about this research is that when the transgenic tuber, modified to express HPV L1 protein, in combination with a novel mucosal adjuvant (LT192G), was ingested by mice, it produced anti-HPV immunogenic responses. This study also showed a humoral immunogenic boosting effect when HPV VLPs were delivered orally, indicating some specific immune response to the delivery of this vaccine to the mucosal immune surfaces. This is important to our research, because if an immune response is observed after vaccination of the gastrointestinal tract, immune response through the pulmonary mucosa may also occur. While VLPs are currently seen to be necessary for successful immunization, several studies under the direction of our collaborator Dr. Robert Garcea have shown that the individual HPV L1 subcapsid particles, or pentamer capsomeres, that show no evidence of self-assembly into VLPs, may have potent immunogenicity (Moscicki 2005). The HPV L1 protein that we have researched is an approximately 300kDa protein, roughly pentagonal in shape with an inner void, appearing much like a "donut" (Syrjanen 2005). These proteins can be economically expressed in and purified from *E. coli* and may be very stable alternatives to VLPs. The HPV L1 capsid proteins have also been shown to induce a strong cytotoxic T-Cell response and show tumor regression in mice (Swaminathan, Selvakumaran et al. 2009). Individual L1 capsid proteins, synthesized from *E. coli* may lead to a second generation HPV vaccine as a stable, economical and effective, alternative to the VLP formulations.

5.2 Inhaled HPV Vaccine Theory

While advancements have been made recently in HPV vaccination, there is still much room for improvement. The cost of a current HPV vaccine series, $300-$500 in 2011, may be considered prohibitive in lower-income or poverty-stricken populations. The extended vaccination series may become a problem particularly in areas without consistent healthcare, where women may not be able to access a health-care worker over the required 6 months. A readily available and easily administered vaccination for HPV could greatly reduce the incidence and high mortality of cervical cancer, particularly in areas where women may not be able to get, or are culturally prohibited from getting, regular pap smears for early diagnosis of cervical cancer. The difficulty of injection (pain, contamination, compliance and disposal of sharps), required training of delivery staff and the 3-dose series, may lead to lower patient compliance and fewer women returning for the subsequent injections. Even in the U.S., compliance with the recommended dosing schedule is spotty, with over 50% of doses delivered late in some areas (Ferlay J and 2010) and up to 45% of girls initiating the vaccination, yet not completing the series (Jemal, Bray et al. 2011). Additionally, the requirement that the liquid vaccines be stored at 2-8 °C limits the geographical range that a mass vaccination campaign can cover. These vaccines cannot be reliably delivered outside the cold chain and may be destroyed by freeze-thaw if the storage conditions are not constantly regulated. This was the primary motivation to develop, test and optimize a dry-powder, L1 capsid subunit-based HPV vaccine for delivery to the mucosal surfaces or for reconstitution and injection.

The goals of our formulation work on a dry powder HPV vaccine have been increased efficacy, ease of delivery and increased patient compliance. This is currently measured in the conservation of protein and immunogenicity throughout processing of fine particle, properties, such as particle size, shape, density and moisture content and the stability of the protein content, activity and particle properties during storage. VLP-based HPV vaccine, formulated for liquid intranasal delivery, has shown an immune response in mice (Giuliano, Palefsky et al. 2011), and immune responses in mice have been observed after intranasal delivery of the L1 capsid proteins (CDC-ACIP 2011). Nasal delivery carries with it several difficulties, including high loss to mucosal filtration, non-uniform delivery due to nasal differences/blockages and limited immune-active surfaces. Therefore, our research the lungs were chosen as the primary targeted route of entry for mucosal delivery of the HPV L1 antigen.

The pulmonary system, a natural target of infectious microbes, contains mucosa-associated lymphoid tissues (Crosbie and Kitchener 2007) and are patrolled by dendritic cells adapted for antigen presentation to T-cells (Widdice and Kahn 2006), conducive to generating good mucosal and humoral immune responses. By delivering microparticles over the large surface the deep lungs, where particle removal is limited by phagocytosis or active transfer across the lung epithelium, the residence time of the vaccine powder can be increased . Additionally, the lower concentration of native proteases in the alveolar fluid will reduced the chance of deactivation of the protein-based vaccine by enzymatic degradation which is a potential concern with oral and sublingual delivery.

As reported previously, pulmonary delivery through inhalation of a wet mist aerosol of live attenuated measles virus has been shown to have effectively immunized over 3 million children in Mexico for measles (Yuan, Estes et al. 2001). Recent research has shown that pulmonary delivered dry powder influenza vaccines (influenza subunit proteins with no adjuvant) in mice induces a mucosal, systemic humoral, and cell-mediated immune response superior to conventional i.m. vaccination (Yuan, Estes et al. 2001). While this study concentrated on mucosal immunity in the airways, the body’s lymphatic system has been shown to communicate immunity between distant lymphoid tissues in the body through the lymphatic system, leading to mucosal immunity in the genital mucosa as a result of immunization in the respiratory tract (Chen, Casini et al. 2001). In a 1998 study by Dr. Carole Balmelli et al, mice given a liquid formulation of HPV-16 VLPs administered nasally under anesthetic showed a significant HPV-16- specific IgG response in the blood. What was particularly noteworthy was that high levels of HPV-16 specific IgG and IgA were also observed in the oral and vaginal mucosa (Ohlschlager, Osen et al. 2003). This mucosal immunity was only noted when the mice were vaccinated under anesthetic, resulting in a significant deposition of the VLPs in the lungs. Mucosal immunity to HPV would benefit the recipient by preventing initial infection and aiding in viral clearance. Mucosal immunity would result in the production of HPV targeting IgA, a class of antibody specific to the mucosa which would inactivate the virus at the point of infection. This additional protection may help to protect women with immunological disorders by producing a more robust immune response. The mucosal (IgA) and humoral (IgG) antibodies present in vaginal mucosa have been noted to fluctuate throughout the estrous cycle, which may mean that the added protection of IgA in addition to IgG may provide more consistent protection. Mucosal immunity in the airways may also reduce the risk of HPV caused throat and neck cancer, increasing the direct value of this vaccine to males, in which HPV-related genital cancer is rare. Additionally, recent studies have shown the presence of HPV16 in certain types of lung cancer (Widdice, Bernstein et al. 2011), further justifying development of a pulmonary vaccine for HPV.

5.3 Formulation

HPV 16 L1 capsomere protein samples were initially obtained from Dr. Garcea’s lab in a buffer L solution consisting primarily of Tris Buffer, NaCl and glycerol. Due to the favorable particle forming properties of *myo*-inositol with l-leucine demonstrated in the preparation of *myo*-inositol powders for previous toxicology tests, this mixture was chosen as the initial particle formulation. The initial HPV powders were prepared and showed retained protein content by SDS-PAGE analysis , but showed poor powder performance and appeared to aggregate excessively, as seen in Figure 5.1. Initial formulation studies concentrated on the removal of components of the Buffer L solution.

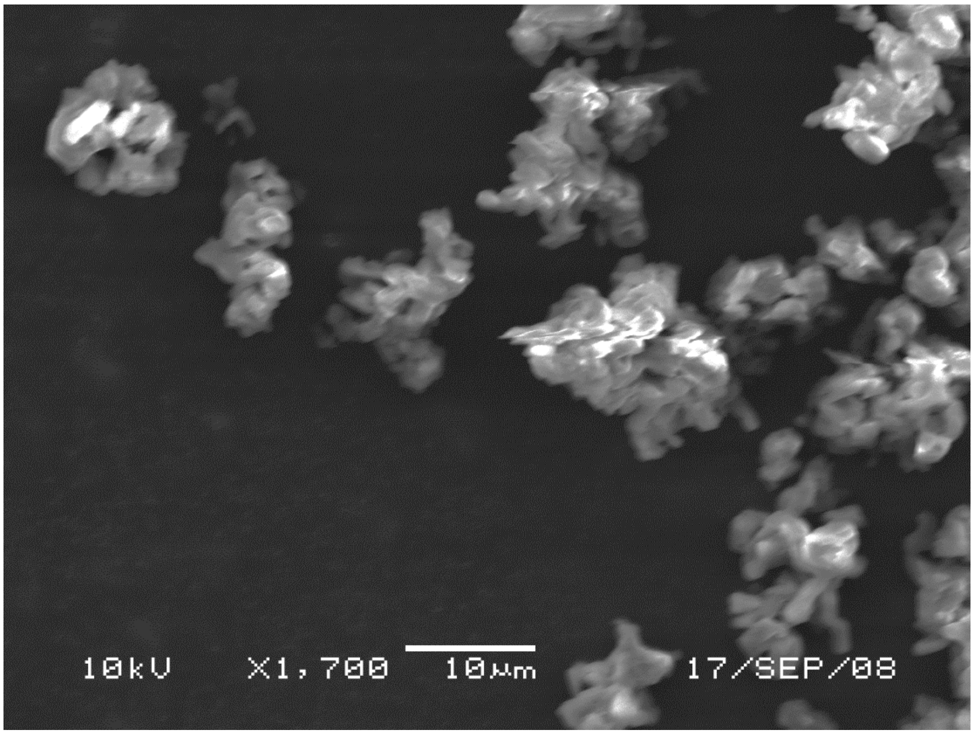

Figure 5.1 SEM images of an aggregated L1 protein containing powder

Initially, glycerol was removed from the protein buffer. Glycerol is a known plasticizer that is liquid at room temperature and is likely to cause particle cohesion and adhesion, by lowering the glass transition of the final powder. One particularly large difficulty in designing a microparticle for delivery of a pharmaceutically active protein is consistency in the delivered dose. The concentrations of the protein received varied between batches and, in order to maintain consistent dose the volume of buffer added to the stock solution for CAN-BD processing was varied. At the concentrations used, between 1 and 10 μg/ml, the protein is not thought to have as much of an effect as the other excipients in the protein buffer, with concentrations up to 10 mg/ml in the initial stock. Efforts were made to produce consistent ratios of buffer to water in the stock solution for processing, and the optimal ratio was determined to be 1 part buffer to 3 parts aqueous solution, in order to maximize delivered dose with the minimum amount of buffer components. Of the buffer ingredients, Tris buffer was considered necessary for protein stability and NaCl was required to prevent protein precipitation. Tris buffer has been noted to result in the collapse of lyophilized material (Niccolai, Mehta et al. 2011). It is believed that during the drying phase high concentrations of salt may reduce the vapor pressure of the liquid droplet, leading to wet particles resistant to drying and likely to stick to one another or the filter upon collection . Several different salt concentrations were processed and reducing salt concentration showed some improvement of powder properties.

Other studies were conducted to maximize protein content of the stock solution by dialyzing the protein into a solution containing *myo*-inositol, l-leucine and the buffer components at roughly 10% TDS. This proved successful in making better-performing powders, in terms of dispersibility and respirable fraction. Large quantities of the buffer/excipient solution were require relative to the resulting stock solution and powder property improvement was not thought to be sufficient to justify the additional dialysis step.

5.3.1 Inclusion of ammonium acetate

Previous studies with spray dried gold nanoparticles, produced by Dr. Feldheim's group, in solution with 8 mM ammonium acetate had shown significant respirable fraction improvement of the *myo*-inositol/ l-leucine powder formed by CAN-BD processing. Particles produced with this formulation appear hollow and show some evidence of burst hollow particles in SEM images, Figure 5.2.

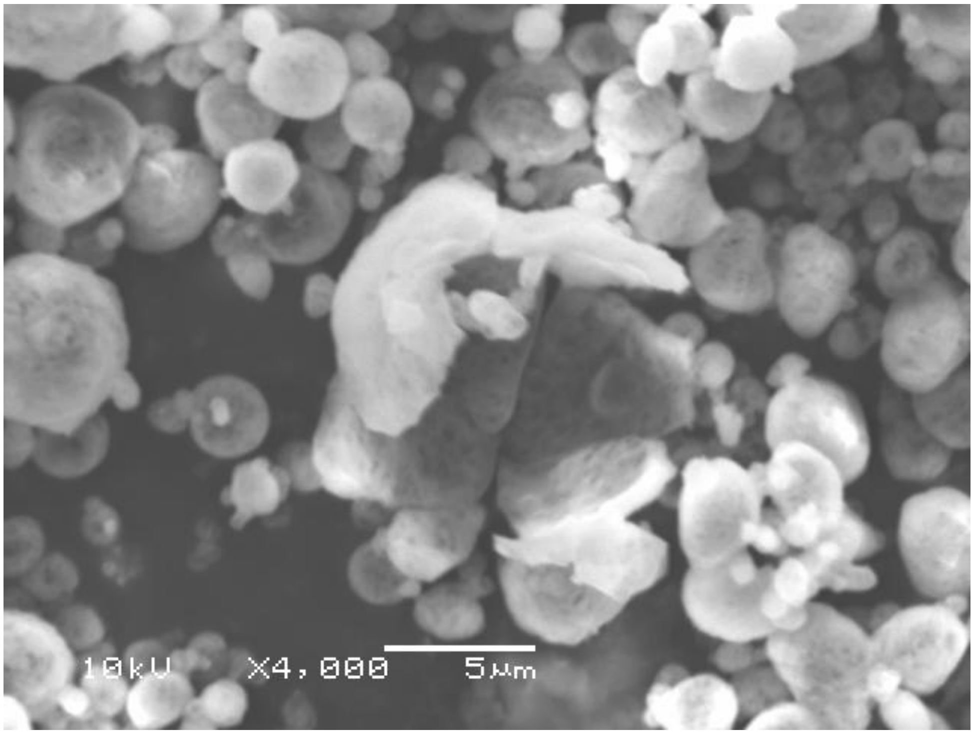

Figure 5.2 Hollow particle of *myo*-inositol/leucine and gold nanoparticles made with 8 mM ammonium acetate stock solutions.

The concentration of gold nanoparticles was assumed to be too small to have an appreciable effect on the particle properties, leading to experiments where ammonium acetate was added to *myo*-inositol/l-leucine stock solutions and processed by CAN-BD. A general increase in respirable fraction was noted in powders formulated with between 0.4% and 1.5% ammonium acetate, with maximum increases of up to 10% in particles below 5.8 μm in aerodynamic diameter and an increase of up to 15% in particles below 3.3 μm in aerodynamic diameter, Table 5.1.

Formulation	Average	±	Average	±
	%<3.3 μm	%<3.3 μm	%<5.8 μm	%<5.8 μm
myo-inositol/leucine (98.5:1.5)	18.3	1.9	56.7	3.5
+				
0.4% $NH_4C_2H_3O_2$	28.9	2.0	63.8	3.0
0.8% $NH_4C_2H_3O_2$	20.6	1.6	50.3	4.0
1.5% $NH_4C_2H_3O_2$	21.9	1.3	56.1	2.9
0.4% $NaC_2H_3O_2$	26.8	1.0	59.2	3.1
0.8% $NaC_2H_3O_2$	23.1	1.9	55.1	2.7
1.5% $NaC_2H_3O_2$	26.6	0.3	70.3	0.6

Table 5.1 Particle size by ACI for different additions of acetate salts in a *myo*-inositol/leucine based powder

In order to determine the source of the improvement powders were prepared with the addition of sodium acetate and showed similar improvements of respirable fraction. Powders formulated with ammonium chloride at the same molar concentrations used in the ammonium acetate studies, did not show improvement; in fact the respirable fraction dropped significantly compared to powder using only *myo*-inositol and l-leucine. This implies that the presence of acetate may be the primary factor in the respirable fraction improvement, but the ammonium ion may be working in concert with the acetate ion. We can propose several explanations for the improvement in the respirable fractions of these powders. The addition of ammonium acetate or sodium acetate may have beneficial pH buffering effects, the ammonium ions may be forming ammonium bicarbonate during processing or the acetate may be interacting beneficially with *myo*-inositol or l-leucine beneficial to fine particle formation.

A solution of ammonium acetate acts as a buffer with a pH of 7. While extensive studies have not been conducted, it is thought that pH may play a role in the quality of powder produced by CAN-BD processing. pH effects may come into play during the emulsification of the aqueous solution with at the mixing tee. The concentration of dissolved carbon dioxide may be closely related to the pH of the solution through the carbon dioxide carbonic acid equilibrium.

$$CO_{2(aq)} + H_2O \leftrightarrow HCO^-_{3(aq)} + H^+_{(aq)} \leftrightarrow H_2CO_{3(aq)}$$

This may be altered by the presence of the acetate or ammonium ion, resulting in differences in the solubility or dissolution of carbon dioxide in the process liquids or the droplets of the spray. Additionally, the pH of the spray solution may influence the solubility of the dissolved solids in the spray droplets. If the dissolved solids are less soluble, a solid skin will form around the drying droplet at an earlier stage (Balmelli, Roden et al. 1998), resulting in a hollow particle of low density and high dispersibility. This is unlikely as pH measurements of the formulation after the addition of each individual component showed that the Tris buffer in the protein solution dominates the pH of the liquid formulation. *myo*-Inositol/leucine placebo formulations prepared with no additives or 11 mM ammonium acetate, sodium acetate or ammonium nitrate showed different pH values with the addition of the additives, but all solutions exhibited a consistent pH of 8.2 after the addition of the tris buffer solution (buffer L).

Another proposed explanation is that during the drying process, solid ammonium bicarbonate may be produced in the drying droplet solution. Ammonium bicarbonate has been added to other spray-dried formulations to improve powder properties (Ohlschlager, Osen et al. 2003). The proposed method of powder improvement is that ammonium bicarbonate will spontaneously decompose to ammonia and carbon dioxide gas and water at temperatures between 30 and 60 °C.

$$NH_4HCO_3 \overset{\Delta}{\Rightarrow} NH_{3(g)} + CO_{2(g)} + H_2O_{(l)}$$

This would produce additional gas inside the drying droplet (beyond the dissolved CO_2) during the drying process. If the solution is sufficiently viscous, the dried particle could maintain a hollow or porous nature. The process of CAN-BD is thought to operate in part, based on the solubility of carbon dioxide in the aqueous solution of the compound or salts of interest. Additional droplet dispersion and a decrease in initial droplet size are thought to lead to smaller dry particles after drying and collection. The addition of ammonium to the stock solution may result in a "reservoir" for dissolved carbon dioxide, as ammonium bicarbonate. This could lead to gas release during the drying phase of the powder formation, rather than immediately at the decompression at the end of the spray restrictor. If the gas were to be released when the drying liquid droplet has a higher viscosity, the porous character could be more conserved in the final dried particle, much like the production of a froth in a viscous solution vs. a dilute solution. It can be seen, in SEM images, that the particles produced by the addition of ammonium acetate are partially hollow or porous (Figure 5.2). The combination of ammonium and acetate may lead to production of more stable ammonium bicarbonate, resulting in the production of gasses beneficial to the final quality of the powder.

Ammonium acetate is commonly used to precipitate particles (Ohlschlager, Osen et al. 2003) and proteins If this is occurring during the atomizing phase of the CAN-BD process it may result in changes in the solubility of *myo*-inositol beneficial to the production of high quality powder. The manner in which these changes would be beneficial is not immediately apparent, and likely complex in nature, but, the reduction of solubility of *myo*-inositol due to *myo*-inositol acetate adduct/ l-leucine interactions could result in the formation of a dry (or viscous liquid) "skin" earlier in the drying process of the drying liquid droplet. This would result in the formation of a larger hollow particle of lower density. Lower density particles would be easier to disperse and may display lower particle aggregation tendencies (Holmgren and Czerkinsky 2005). Lower density particles will have a lower surface area to mass ratio, leading to weaker particle bonding and the larger diameter could make the particles more likely to interact with a turbulent dispersal air flow.

5.3.2 Final Formulation

The formulation of the HPV protein powder in aqueous solution used in our more advanced experiments contained ammonium acetate at roughly 11 mM, with a total dissolved solids content of 110 mg/ml consisting of the components displayed below and resulting in a powder with the properties listed in the Table 5.2.

material	concentration in liquid	concentration in dry powder
	mg/ml	% (mass/mass)
***myo*-inositol**	100.3	91.6
l-leucine	1.6	1.4
ammonium acetate	0.9	0.8
NaCl	2.8	2.5
Tween	2.4	2.2
Tris Buffer	1.4	1.3
DTT	0.1	0.1
EDTA	0.1	0.1

Table 5.2 Components of L1 protein based HPV vaccine powder

Protein concentrations varied between 1 to 5 μg/mg dry powder, depending on the concentration in the provided protein sample. It should be noted that in lyophilized formulations, both Tris buffer and sodium acetate are known to cause collapse of the lyophilized cake near their glass transition states of -81°C and 80 °C, respectively (Lambrecht, Prins et al. 2001). This may have resulted in the production of poor performing particles; however, this difficulty was overcome by the addition of excipients with high glass transition temperatures in the formulation.

Powders were processed using standard CAN-BD processing conditions with an average drying gas temperature of 70 °C, to avoid the formation of the alternate polymorph of *myo*-inositol at lower temperatures. Powder was collected in glass vials from the Nylon filter by scrapping ,with metal spatulas, in a low humidity (<15% RH) environment. The vials were sealed with molecular sieve desiccant packets in aluminum foil-polymer laminate film overwrap.

Placebo powder used in testing was prepared using this formulation with bovine serum albumin (BSA) at about 1.7 μg/mg dry powder. BSA is a common protein model that has been shown to be well-tolerated when delivered by inhalation to rats (Folkesson et al 1992). Placebo powder without this added protein was not analogous to the active powder and was observed to have different physical properties (Figure 5.4) than the active HPV protein powder (Figure 5.3). This implies additional beneficial effects due to the addition of a low concentration of protein, possibly due to enhanced "skin" formation of the drying particle, leading to hollow particles.

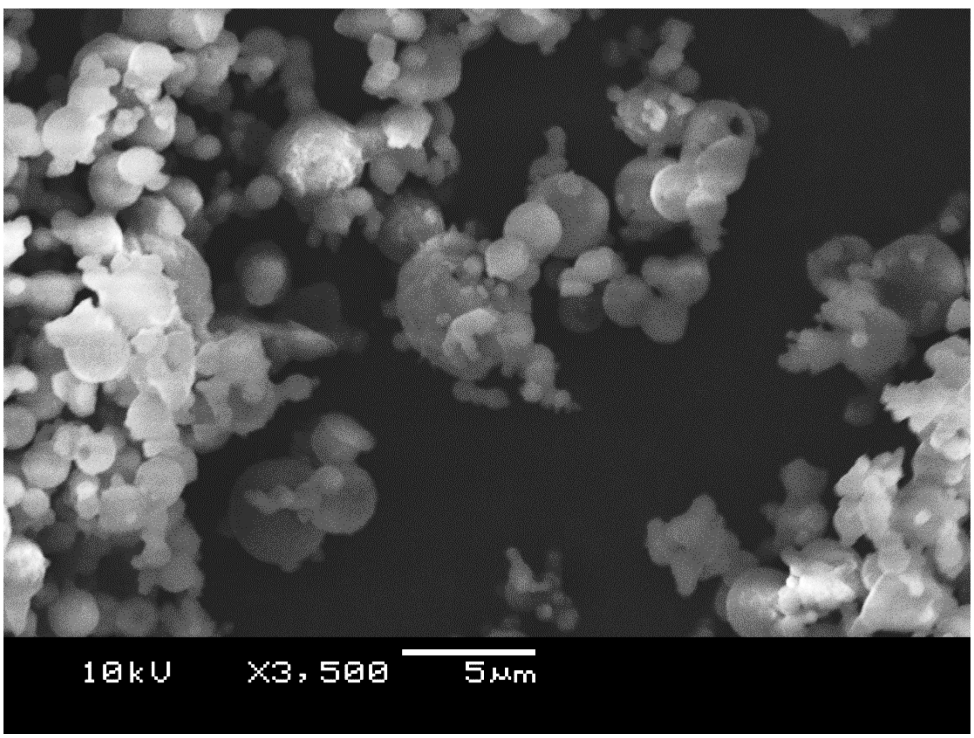

Figure 5.3 CAN-BD processed HPV L1 protein powder

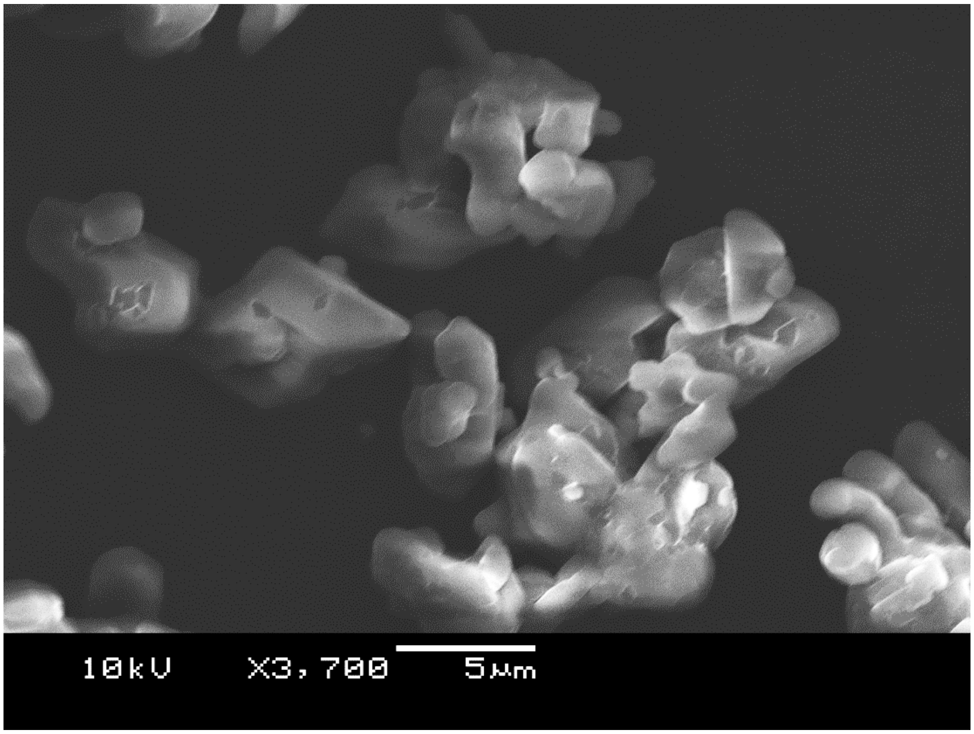

Figure 5.4 CAN-BD processed placebo powder with no protein

It can be seen in Figure 5.3 that the protein powder did have some hollow properties, as shown by holes in some particles and the "collapsed" appearance of some of the larger particles. The powder without the addition of protein exhibited unique morphology with some angled particles that may imply some crystalline content.

The HPV L1 protein containing powder was tested for protein content by SDS-PAGE and for immunogenicity by ELISA using HPV16 L1 specific antibodies by the Garcea research group, Figure 5.5.

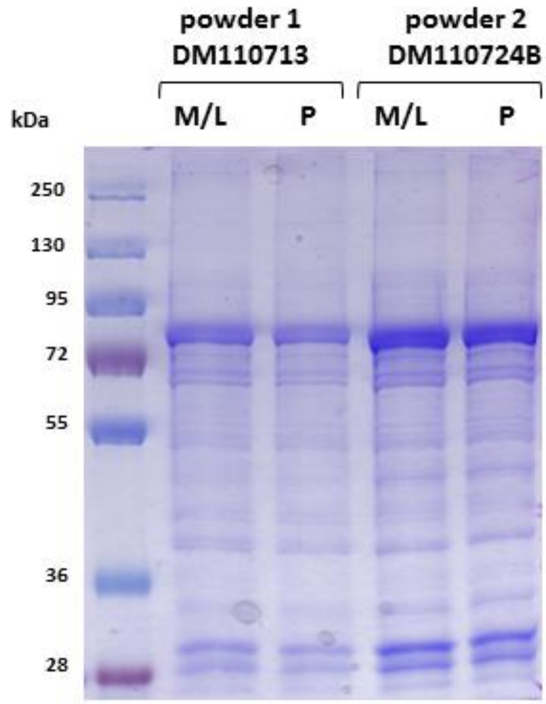

Figure 5.5 SDS PAGE gel (prepared by Dr. Elizabeth Gersh of the Garcea lab) of reconstituted CAN-BD processed L1 protein powder vs L1 stock

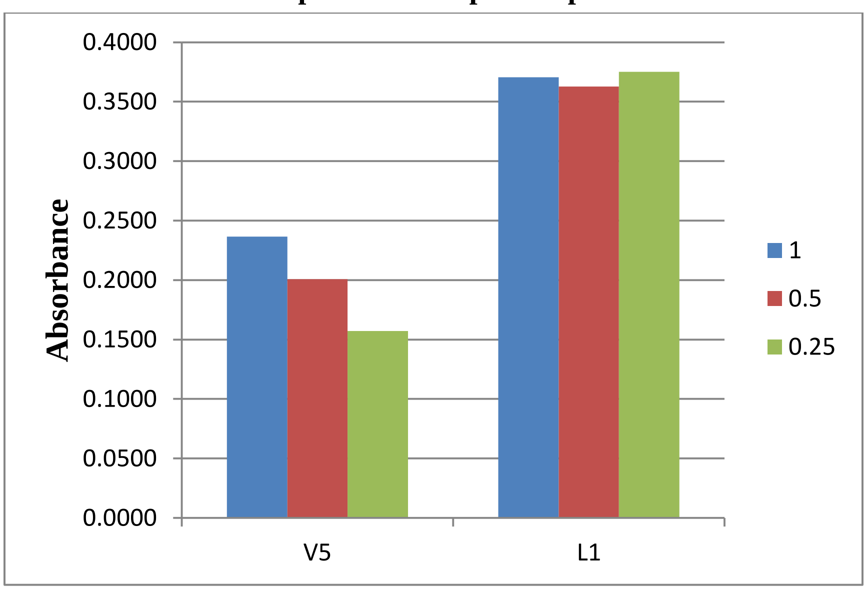

Figure 5.6 Results of ELISA testing of reconstituted CAN-BD processed L1 protein powder (P) and 10% *myo*-inositol/l-leucine solution with added stock L1 protein at the same concentration.

In Figure 5.5 it can be seen that the bands for the CAN-BD processed L1 protein powder are identical to the bands observed for the stock protein in a 10% *myo*-inositol/l-leucine solution. The protein concentration of the positive control (M/L) was determined based on the theoretical concentration of the reconstituted protein powder, based on the initial amount of added L1 protein. The nearly identical intensities of the bands demonstrated excellent conservation of protein content through processing. ELISA testing (Figure 5.6) tested positive for the L1 and V5 epitopes of the HPV16 L1 protein. This shows the presence of the L1 protein with the correct folding (V5) for antigenic response.

HPV16 L1 capsomere specific ELISA testing was conducted on a sample powder and showed good conservation of immunogenicity through processing, providing promising evidence that we had formulated an active dry powder formulation of HPV16 L1 protein antigen. Ongoing studies in Sprague-Dawley rats will determine the immunogenicity of this powder delivered by pulmonary insufflation and reconstitution with i.m. injection.

5.4 *in vivo* Immunogenicity Test Design

Sprague Dawley rats, while not naturally susceptible to Human Papillomavirus serotype 16, were chosen as an appropriate animal model because of the extent of characterization and common use as an animal test model. Initial studies suggested that larger, 11-12 week old animals would be required for dosing by insufflation, with females weighing more than 250 g and males weighing more than 350 g . A total of 32 animals (16 male/16 female and divided evenly among the groups) were included in the test, divided into the groups listed in Table 5.3.

Group	Number	mg Powder Dose	μg L1 Protein Dose
Insufflation Pilot Low Dose	2	1 mg placebo	n/a
Insufflation Pilot High Dose	2	2 mg placebo	n/a
Insufflation Low Dose	4	1	2.4
Insufflation High Dose	4	2	4.8
Sublingual Low Dose	4	1	2.4
Sublingual High dose	4	2	4.8
Injection Low Dose	4	1	2.4
Injection High Dose	4	2	4.8
Injection Control	4		Dilute Gardasil
Total	32		

Table 5.3 Powder testing group assignments

All animal studies were conducted at High Q Research, Ft. Collins CO (HQR) and all animal handling was conducted by HQR facility staff. The animal testing procedure was approved by the facility's IACUC panel. Tail bleed samples were collected before dosage and at designated time points after initial dosing up to a final bleeding and sacrifice at study day 60. At least 5 minutes before dosage, animals were anesthetized by injection with ketamine/xylazine, dosed according to animal weight by the facility veterinarian. The immunogenicity experiment was designed such that animals received an initial dosing on study day 0 and a second dosing on study day 20. An immunogenic response is indicated by the presence of HPV16 specific antibodies in blood samples after dosage and an increased production of HPV16 specific antibodies after the second delivery.

A Model DP-4 Dry Powder Insufflator (PennCentury) was initially loaded with about 6 mg of the powder of interest and dispersed into a contained vessel. This acted to prime the device, providing an initial powder coating of the loading chamber, which, from experience, improved the powder delivery. Immediately before delivery, the empty device was weighed and loaded with 6 mg of the powder of interest. Anesthetized animals were immobilized on an angled board, a drop of lidocaine was placed on the vocal cords to prevent tracheospasm. HQR staff then placed the loaded insufflation device into the trachea with the device tube opening about 1-3 mm from the initial airway branching. HQR staff quickly dispensed 1 mL of air from a 3 mL syringe after which, the device was removed, weighed, and cleaned. The mass of powder delivered was determined by the difference between the loaded weight of the device and the weight of the device after delivery.

5.4.1 Placebo Insufflation Pilot Group

An initial pilot study was conducted to prepare a more informed insufflation technique and determine tolerance of the animals for insufflated powder. This will also serve as a *de facto* negative control group. Powder Placebo containing BSA was dispersed directly into the lung of the animals using the previously stated insufflation method. Desired dosage was 1 mg powder for the low dose group and 2 mg powder in the high dose group as the range of previously insufflated powder dosage with rats has been reported to be between 1 mg and 5 mg (Valdespino-Gomez, Garcia-Garcia et al. 2006). Variabilities in the powder and delivery device led to a larger range of powder delivery in the pilot group. Animals received 0.5 mg to 4.1 mg of placebo powder insufflated into lungs, with no immediate or subsequently observed adverse effects. Animals were dosed on pilot study Day 0 and again on pilot study Day 20, and were sacrificed on pilot study Day 60. The pilot test showed that, while it was difficult to deliver a consistent dose, Sprague-Dawley rats could tolerate doses up to 4.1 mg of insufflated placebo powder with no detectable adverse effects.

5.4.2 HPV16 L1 Protein Powder Insufflation Group

Insufflation of the active powder was conducted in the same manner as the placebo group. The active HPV L1 protein powder used was DM110724, formulated with *myo*-inositol, l-leucine and trace ammonium acetate with a HPV16 L1 capsomere protein dose of 2.4 μg/mg . This powder had been tested by ELISA in the Garcea lab and showed appropriate immunogenicity. Test animals received the powder with no observed adverse effects. Powder was delivered to the High Dose (at least 2 mg) and Low Dose (at least 1 mg) groups using the insufflation method previously reported. Unfortunately, due to method variability, the powder delivered by insufflation was not exactly within these ranges, powder delivered and protein doses are shown in Table 5.4. The powder was insufflated directly into the trachea with no observed adverse effects.

	Day 0	Day 0	Day 20	Day 20
Group	**Powder Dose** mg	**Protein Dose** µg	**Powder Dose** mg	**Protein Dose** µg
Low	1.1	2.6	3.3	7.9
Low	0.7	1.7	2.8	6.7
Low	3.8	9.1	2.2	5.3
Low	1.4	3.4	2.7	6.5
High	1.6	3.8	2.2	5.3
High	3.3	7.9	2.7	6.5
High	4.4	10.6	3.0	7.2
High	2.0	4.8	2.0	4.8

Table 5.4 Dosage of HPV16 L1 protein powder insufflation group.

5.4.3 HPV16 L1 Protein Sublingual Pellet

Pellets, or wafers, for sublingual delivery were formulated with 1 mg (Low Dose) or 2 mg (High Dose) of DM110724, formulated with *myo*-inositol, l-leucine and trace ammonium acetate with a HPV16 L1 capsomere protein dose of 2.4 µg/mg, bulked with placebo powder and a cellulose-based excipient to a total mass of about 10 mg. Pellets were formed by compression in a pellet press at a deliberately increased relative humidity (40% RH) to make compacted pellets with sufficient cohesion. During testing, pellets were placed under the tongues of anesthetized rats. One drop of water was added to the powder at 1 minute and 2 drops were added at 2 minutes with addition drops of water added at 15 minutes after delivery. The pellets were observed to form a thick slurry under the tongue and were left in the animal’s mouth when they were returned to their cages. No direct adverse reactions were noted.

5.4.4 HPV16 L1 Protein Powder Reconstitution and Injection

Powder was reconstituted in a dilute mixture of Alhydrogel 85 and sterile saline. The diluent was designed to contain the amount of alum adjuvant comparable to the diluted Gardasil in the control group (22.5 μg/mL). The diluent was prepared by diluting 0.25 mL Alhydrogel 85 to 10 mL using sterile saline and then diluting 1 mL of this stock to 10 mL with sterile saline. Stock diluents were shaken well before mixing and the final diluent was well agitated before adding them to the powder. Powder for reconstitution, DM110724, formulated with *myo*-inositol, l-leucine and trace ammonium acetate with a HPV16 L1 capsomere protein dose of 2.4 μg/mg, was packaged into sterile Eppendorf tubes with 10 mg for the Low Dose and 20 mg for the High Dose. After diluent was added, the container was lightly agitated until the powder was completely dissolved. The reconstituted powder was allowed to sit at room temperature for at least 1.5 hours before injection. Both doses appeared to be slightly cloudy and contain distinct cloudy aggregates which settled when not thoroughly agitated. The reconstituted suspensions were shaken well and transferred to the veterinarian who injected 0.1 mL into the thigh of the anesthetized animals. The dose was designed such that the animals would receive 2.4 mg of the HPV16 L1 capsomere protein in the Low Dose group and 4.8 mg of the HPV16 L1 capsomere protein in the High Dose group. One test animal in the Low Dose injection group was found dead after the testing on Day 0; this was attributed by the veterinarian to a reaction to the anesthesia. No other animals showed immediate, or detectable long-term, adverse reactions to injection.

5.4.4 Gardasil Positive Control Group

A control group received an i.m. injection of a 1:20 dilution of Gardasil with sterile saline. The dilution was prepared by agitating the Gardasil vial and diluting 0.25 mL of the Gardasil with 0.75 mL of sterile saline. The diluted suspension was used within 30 minutes of dilution. The tube was shaken well and transferred to the veterinarian who injected 0.1 mL into the left thigh of the animal. There was only one dose group for the Control testing. The dosing was designed to deliver 2 μg of the HPV16 VLP protein, comparable to the low Dose of our reconstituted powder. No adverse effects were noted in any of the animals in the control group. The Gardasil positive control group should give an immune response reference, using an FDA approved HPV 16 vaccine with proven efficacy, for comparison with the powder test groups.

5.4.5 Study Preliminary Results

No results of blood assays are available at the time of the writing of this dissertation, but they should be forthcoming in several weeks after final sacrifice on day 60. The powder, containing *myo*-inositol, l-leucine, ammonium acetate, HPV16 L1 capsomere protein and buffer components was well tolerated by insufflation (up to 4.4 mg), sublingual delivery and delivery after reconstitution with alum adjuvant. No immediate adverse effects or reactions were observed and the results of the immunogenic blood tests are eagerly anticipated and will be published when thoroughly analyzed.

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Conclusions

Vaccines have served to greatly increase the longevity and quality of human life, but delivery by injection carries inherent dangers and costs. Vaccination through the pulmonary inhalation route has been shown to provide good immune protection and warrants further research. Our research has developed an effective and stable dry powder measles vaccine for inhalation produced by the CAN-BD process. We have developed various methods of characterization for this powder to optimize running parameters and observe stability.

Powder X-ray diffraction and Differential Scanning Calorimetry analysis show that the final vaccine formulation was an amorphous glass. NMR comparison of different batches have revealed differences and aided in optimizing preparation procedures. A method of determining the viral activity in particles of different sizes was developed and used to show that the powder activity was homogenous in the particle size ranges important for lung deposition.

Pure *myo*-inositol powder was produced by the CAN-BD process and found to be crystalline. Analysis of this crystal powder shows the existence of two anhydrous crystalline forms, one representative of the expected crystalline structure and one with no previously reported pXRD diffraction pattern, produced by different process parameters. The novel polymorph was shown to be metastable and recrystallize into the native form when heated to 145 ºC or stored at room temperature for extended periods of time. A powder containing *myo*-inositol and l-leucine was prepared for toxicity testing and shown to be well-tolerated when delivered to rats by at-liberty breathing.

With the goal of reducing the global mortality and morbidity due to Human Papillomavirus infection, and the resulting cervical, penile, head and neck and other cancers, a dry powder HPV protein subunit vaccine was developed. Protein material was provided by Dr. Robert Garcea;s lab. The *myo*-inositol based formulation was designed and improved with the addition of ammonium acetate in low concentrations. The powder was shown to retain protein content and the proper epitope for antigenic activity by SDS-PAGE and ELISA testing. This powder was used in immunogenicity tests with Sprague Dawley rats and shown to be well tolerated when delivered by insufflation, sublingual wafer and injection after reconstitution with alum adjuvant. The results of this immunogenicity test are forthcoming at the time of the writing of this dissertation and will be published once they have been collected and analyzed.

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Appendix A Selected Publications/Posters/Patent Applications:

Publications

1. "An Inhalation Safety Study of Myo-inositol, A Non-Hygroscopic Stabilizing Excipient in Dispersible Microparticles, D.H. McAdams, S.P. Cape, W.E. Winston, S. Godin, K.G. Powell, C.D. Shermer, L. Chan, P. Pathak, R.E. Sievers, *Journal of Aerosol Medicine and Pulmonary Drug Delivery*, 22:2, 199, 2009.

2. "Are Unit-dose Dry Powder Vaccines Intrinsically Safer than Liquid Vaccines, R.E. Sievers, E.L. Sievers, J.A. Searles, S.P. Cape, D.H. McAdams, J.L. Burger, J.R. Manion, D. Griffin, W-H Lin, P. Rota, M. Papania, S. Winston, B.P. Quinn, D.M. Krank, P. Pathak, P.A. Bhagwat, L.G. Rebits, S. Evans, *J. Pharmacy and Pharmacology,* Supplement 1 A163-164, September 2009.

3. "Myo-inositol as a Stabilizing Excipient for Inhaled Dry Aerosol Vaccines and Pharmaceuticals," McAdams, D.H., S.P. Cape, J.L. Burger, R.A. Kudgus, D.L. Feldheim, S.E. Winston, K.G. Powell, S. Godin, J.A. Searles, D.M. Krank, P. Pathak, L.G. Rebits, B.P. Quinn, R.E. Sievers, *Respiratory Drug Delivery Europe 2009*, 2 399-404, isbn: 1-933722-31-2, 2009.

4. "Packaging Unit Dose Inhalable Dry Powder Formulations to Protect Against Moisture Ingress," Powell, K.G., L. Chan, C.D. Shermer, D.H. McAdams, S.P. Cape, J.L. Burger, B.P. Quinn, D.J. Bennett, L.G. Rebits, J.A. Searles, R.E. Sievers, *Respiratory Drug Delivery Europe 2009* 2 245-250, isbn: 1-933722-31-2, 2009.

5. "Challenges of Developing a Stable Dry Powder Live Viral Vaccine," R.E. Sievers, S.P. Cape, K.O. Kisich, D.J. Bennett, C.S. Braun, J.L. Burger, J.A. Best, D.H. McAdams, N.A. Wolters, B.P. Quinn, J.A. Searles, D.M. Krank, P. Pathak, P.A. Bhagwat, L.G. Rebits, *Respiratory Drug Delivery 2008*, Vol. 1, 281-290, 2008.

6. "The PuffHaler®: a Simple Active DPI with a Pressure Release Valve Dispenser," D.J. Bennett, R.E. Sievers, S.P. Cape, J.A. Best, A.L. Morin, C.A. Pelzmann, B.P. Quinn, L.G. Rebits, S. Evans, R.D. Threadgill, D.H. McAdams, *Respiratory Drug Delivery 2008*, Vol. 2, 345-350, 2008.

7. "Stabilizing Formulations for Inhalable Powders of Live-Attenuated Measles Virus Vaccine," J.L. Burger, S.P. Cape, C.S. Braun, D.H. McAdams, J.A. Best, P. Bhagwat, P. Pathak, L.G. Rebits, R.E. Sievers, *Journal of Aerosol Medicine and Pulmonary Drug Delivery*, **21**:1, 25-34 (March 2008)

Posters

1. "Development of Needle-Free Vaccines: Dry Powder for Inhalation and Wafers for Sublingula Delivery" S.P. Cape, D.H. McAdams, D.J. Chen, H.K. Shah, P. Pathak, S.E. Winston, B.P. Quinn, R. Meesala, S.V. Kapre, R.M. Dhere, V.B. Vaidya, R.G. Muley, P.S. Kulkarni, P.J. Barde, P.M. Akut, S. Gairola, D.E. Griffin, W.-H. Lin, C.S. Godin, P.A. Rota, M. Papania, K.G. Powell, C.D. Shermer, and R.E. Sievers, 7th Annual GCGH Meeting; New Delhi, India, 4 - 7 November 2011

2. "Development of Sublingual Vaccine Dry Wafer Formulations" S.P. Cape, R.A.

Rodriguez, D.J. Chen, N.K. Shah, H. L. Richter, D.H. McAdams, J.A. Manion, M.T. Hernadez, S.E. Winston, and R.E. Sievers, the 2011 American Association of Pharmaceutical Scientists (AAPS) Annual Meeting and Exposition; Washington, DC, 23 - 27 October 2011

3. "Development of Stable, Immunogenic, and Protective Measles Vaccine for Needle-Free Administration," S.P. Cape, D.H. McAdams, J.R. Manion, SR.G. Muley, V. Vaidya, R.M. Dhere, P.A. Bhagwat, P. Pathak, J.A. Searles, D.M. Krank, S.C. Evans, S.E. Winston, B.P. Quinn, D.E. Griffin, W-H. Lin, P.A. Rota, M.J. Papania, C.S. Godin, R.E. Sievers, 2010 FIP Pharmaceutical Sciences World Congress and AAPS Annual Meeting and Exposition, New Orleans, LA, November 14-18, 2010.

4. "Production of an Inhalable Dry Powder HPV Vaccine by CAN-BD," D.H. McAdams, S.P. Cape, E. Frederick, R.L. Garcea, R.E. Sievers, 2010 FIP Pharmaceutical Sciences World Congress and AAPS Annual Meeting and Exposition, New Orleans, LA, November 14-18, 2010.

5. "Development of a Needle-Free, Inhalable, Mucosal Measles Vaccine," R. E. Sievers, R.M. Dhere, P.S. Kulkarni, V.B. Vaidya, R.G. Muley, S.P. Cape, D.H. McAdams, J.R. Manion, S.C. Evans, P. Pathak, S.E. Winston, B.P. Quinn, R. Meesala, D.E. Griffin, W-H. Lin, P. A. Rota, C.S. Godin, Keystone Meeting, Seattle, Washington, October 27-30, 2010.

6. "Stabilization, Synthesis, Drying and Characterization of Microparticles for Inhalation and Sublingual Delivery of Measles and HPV Vaccines," S.P. Cape, R. Meesala, J.R. Manion, D.H. McAdams, M.W. Howard, R.L. Garcea, E. Frederick, J.A. Searles, D.M. Krank, D.J. Bennett, P. Pathak, S.C. Evans, L.G. Rebits, S.E. Winston, R.G. Muley, V.B. Vaidya, R.M. Dhere, D.E. Griffin, W-H. Lin, P.A. Rota, M. Papania, K.G. Powell, C.D. Shermer, and R.E. Sievers, 4th Vaccine Congress, Vienna, Austria, October 3-5, 2010.

7. "Characterization of myo-Inositol as a Particle-Forming and Stabilizing Excipient for Inhalable Measles and Human Papillomavirus Vaccines," D.H. McAdams, S.P. Cape, R.L. Garcea, R.E. Sievers, RDD 2010, Orlando, FL, April 25-29, 2010.

8. "Inhalable Dry Powder Live-Virus Measles Vaccine Prepared by the CAN-BD Process, a Novel Spray Drying Alternative to Lyophilization," S.P. Cape, D.H. McAdams, J.R. Manion, J.L. Burger, M.W. Howard, L.G. Rebits, P.A. Bhagwat, P. Pathak, D.M. Krank, J.A. Searles, S. Winston, B.P. Quinn, R.E. Sievers, CHI PEPTalk, Coronado, CA, January 10-15, 2010.

9. "An Inhalation Safety Study of Myo-inositol, A Non-Hygroscopic Stabilizing Excipient in Dispersible Microparticles, D.H. McAdams, S.P. Cape, W.E. Winston, S. Godin, K.G. Powell, C.D. Shermer, L. Chan, P. Pathak, R.E. Sievers, RDD Europe, Lisbon, Portugal, May 19-22, 2009.

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